UNIVERSITY OF ALBERTA

DEVELOPMENT OF A MULTIPLE PEPTIDE SYNTHESIZER AND COMPLEMENTARY MULTIPLE PEPTIDE PURIFICATION SYSTEM

by

Devon Leigh Husband ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta
Spring 1999

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Degree:	Doctor of Philosophy
Year this Degree Granted:	1999

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ABSTRACT

A simple, inexpensive semi-automated synthesizer and complementary peptide purification system have been designed to bring multiple peptide synthesis to general laboratory use. The synthesizer employs two versions of a dual-purpose reaction module containing an array of embedded column reactors to perform parallel synthesis and cleavage: a 20-reactor model conveniently designed for general use and construction of analogue series, and a 100-reactor model for large numbers of peptides and creation of peptide libraries. The novel valveless module uses positive pressure for maintaining solutions within reactors, aspiration for evacuation, and sparging for mixing. Solid-phase synthesis is based on Fmoc strategy; activated coupling solutions are added to reactors manually (permitting flexibility in synthesis chemistries for different reactors), while common washing, deprotection and cleavage steps are automated by a novel dispensing station for simultaneous delivery to reactors. A standard protocol for new practitioners provides consistent yield and purity over a range of synthesis scale.

A novel combination of sample displacement chromatography (SDC) methodology and solid-phase extraction (SPE) technology provides a simple, rapid and inexpensive process for simultaneous peptide purification under low pressure conditions for a range of peptide load, hydrophobicity and impurities. The main separation process occurs in the absence of organic modifier, greatly reducing solvent consumption and operating cost. The system employs two-stage purification units (formed from SPE cartridges packed with silica-based reversed-phase stationary phase) mounted on a vacuum-operated SPE processing station; each unit consists of a pre-column acting as a trap for hydrophobic impurities in series with a product isolation column. Crude peptide is applied in 0.05% aqueous trifluoroacetic acid at overload

conditions; hydrophobic impurities remain on the trap while product is displaced onto the product isolation column and hydrophilic impurities are displaced from the column to waste, and pure product is subsequently recovered from the column by elution with 50% aqueous acetonitrile. A reference set of model synthetic peptides spanning a range of hydrophobicity has been designed to determine key parameters for the development of standard purification protocols.

ACKNOWLEDGEMENTS

Bob Hodges, my supervisor, for his support and guidance.

Colin Mant and Lorne Burke for their invaluable technical assistance in the field of purification, and likewise Randy Irvin and Wah Wong in the field of immunological screening.

Bob Parker, Jack Moore, Terri Keown, and Mike Carpenter in the Alberta Peptide Institute for the benefit of their expertise and the use of their equipment.

Jonathan Tyler and his staff at Tyler Research Corporation, for their skill in all aspects of scientific equipment design.

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LIST OF ABBREVIATIONS

Amino acids:

Ala (A)	alanine
Arg (R)	arginine
Asn (N)	asparagine
Asp (D)	aspartic acid
Cys (C)	cysteine
Gln (Q)	glutamine
Glu (E)	glutamic acid
Gly (G)	glycine
His (H)	histidine
Ile (I)	isoleucine
Leu (L)	leucine
Lys (K)	lysine
Met (M)	methionine
Phe (F)	phenylalanine
Pro (P)	proline
Ser (S)	serine
Thr (T)	threonine
Trp (W)	tryptophan
Tyr (Y)	tyrosine
Val (V)	valine

2-BrZ	2-bromobenzyloxycarbonyl
2-ClZ	2-chlorobenzyloxycarbonyl
2,6-Cl_2Bzl	2,6-dichlorobenzyl
Ac	acetyl
Acm	acetamidomethyl
ACP	acyl carrier protein
ACTH	adrenocorticotropin hormone
AOP	(7-azabenzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
BHA	benzhydrylamine resin
Boc	t-butyloxycarbonyl
Bom	benzyloxymethyl
BOP	(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexfluorophosphate
Bum	t-butoxymethyl
Bzl	benzyl ether
cAMP	cyclic adenosine monophosphate
cdrill	counter drilled
CHO	formyl
DARPP-32	dopamine- and cAMP-regulated phosphoprotein
DCC	N,N'-dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	N,N'-dicyclohexylurea
DIA	diameter

DIEA	N,N-diisopropylethylamine
DIPCDI	N,N'-diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMS	dimethylsulfide
Dnp	2,4-dinitrophenyl
DVB	divinylbenzene
EDT	1,2-dithioethane (ethanedithiol)
ELISA	enzyme-linked immunosorbent assay
EQ SP	equally spaced
Fmoc	9-fluorenylmethyloxycarbonyl
G_L	liver glycogen binding protein
G_M	glycogen binding protein
HAL	5-(4-hydroxymethyl-3,5-dimethoxyphenoxy)valeric acid handle
HAPyU	1-(1-pyrrolidinyl-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene)-pyrrolidinium hexafluorophosphate N-oxide
HATU	N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide
HBTU	N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methyl-methanaminium hexafluorophosphate N-oxide
HF	hydrogen fluoride
HIV	human immunodeficiency virus
HMPA	4-hydroxymethylphenoxyacetic acid handle
HMPB	4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid handle

HOAt	7-aza-1-hydroxybenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
I_{50}	peptide concentration required for 50% inhibition of binding
I-1	inhibitor protein-1
I-2	inhibitor protein-2
ID	inner diameter
IRAA	internal reference amino acid
LHRH	luteinizing hormone releasing hormone
M_{110}	myofibrillar binding protein
MBHA	4-methylbenzyhydrylamine resin
Meb	4-methylbenzyl
MPS	multiple peptide synthesis
Mtr	4-methoxy-2,3,6-trimethylbenzenesulfonyl
Mts	mesitylene-2-sulfonyl
NIPP-1	nuclear inhibitor of protein phosphatase-1
NMM	N-methylmorpholine
Nonb	3-nitro-4-aminomethylbenzoic acid handle
O	sulfoxide
O-1-Ada	1-adamantyl
O-2-Ada	2-adamantyl
OAt	7-aza-1-hydroxybenzotriazole active ester
OBt	1-hydroxybenzotriazole active ester

OBzl	benzyl ester
OcHx	cyclohexyl ester
OD	outer diameter
ODhbt	1-oxo-2-hydroxy-dihydrobenzotriazine active ester
ONb	3-nitro-4-hydroxymethylbenzoic acid handle
OPfp	pentafluorophenyl active ester
OtBu	t-butyl ester
p110Rb	retinoblastoma protein
PAL	5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid handle
PAM	4-hydroxymethylphenylacetic acid handle
PEG	polyethylene glycol
PKA	cAMP-dependent protein kinase
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl
PP-1	protein phosphatase-1
PP-1c	protein phosphatase-1 catalytic subunit
PPG	polypropylene glycol
PTFE	polytetrafluoroethylene (teflon)
PyAOP	(7-azabenzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate
PyBOP	(benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate
RP	reversed phase
RPCL	representative peptide combinatorial library
RP-HPLC	reversed phase HPLC
SAMPS	semi-automated multiple peptide synthesizer

SDC	sample displacement chromatography
SDM	sample displacement mode of HPLC
SOP	standard operating procedure
SPCL	soluble peptide combinatorial library
SPE	solid phase extraction
SPPS	solid-phase peptide synthesis
SS	stainless steel
TATU	N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium tetrafluoroborate N-oxide
TBTU	N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium tetrafluoroborate N-oxide
tBu	t-butyl ether
TEA	triethylamine
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulfonic acid
$Tl(Tfa)_3$	thallium trifluoroacetate
Tmob	2,4,6-trimethoxybenzyl
TMSOTf	trimethylsilyltrifluoroacetate
Tos	4-toluenesulfonyl (tosyl)
Trt	triphenylmethyl (trityl)
V_{col}	Total volume of solvated product isolation column packing bed
V_{trap}	Total volume of solvated hydrophobic trap packing bed
V_{unit}	Total combined volume of solvated trap and column packing beds
V_{tube}	SPE tube volume
XAL	5-(9-aminoxanthen-2-oxy)valeric acid handle

Xan	9-xanthenyl

Chapter 1 INTRODUCTION

We are indebted to the development of solid-phase peptide synthesis for the ready availability of synthetic peptides as tools in biological and medical research . The replacement of difficult, time-consuming, and labour-intensive solution synthesis with a more tractable solid-phase method amenable to automation, along with improvements in synthesis chemistries, purification, and analytical techniques, have made synthetic peptides accessible to investigators in the field of protein research. Application of these synthetic peptides to classical protein structure-function studies has significantly improved our understanding of peptide and protein structure and conformation.

This knowledge, in combination with rapid advances in molecular biology and immunology, is now suggesting new approaches and expanding opportunities for the application of designed synthetic peptides to a wide range of problems in biology and medicine. For example, peptide substrates of kinases, phosphatases, and proteases are now used not only in the isolation and study of enzymes, but also in the design of enzyme inhibitors. Synthetic analogues of peptides functioning as hormones, growth factors, cytokines, and neurotransmitters are used as tools to investigate the physiology and pharmacology of these peptides, and radio- and affinity-labeled analogues are used in receptor type and subtype characterization and isolation. The potential of peptide-based therapeutics is apparent: several peptide hormones and their synthetic analogues have already found use, including vasopressin, oxytocin, LHRH (luteinizing hormone releasing hormone), ACTH (adrenocorticotropin hormone), and calcitonin, and peptide inhibitors of a number of enzymes are in use or under investigation, most notably HIV (human immunodeficiency virus) protease.

Peptides can be employed as synthetic antigens for the preparation of antibodies targeted to specific protein sequences. Putative gene products are commonly assigned and characterized using anti-peptide antibodies raised against synthetic fragments of the predicted protein sequence, and this technique has special application to the detection of protein processing intermediates. Protein antigenicity is routinely studied by epitope mapping with small (6 to 10 residue) synthetic protein fragments, and conformational analysis of antigenic peptide analogues provides information on molecular recognition processes between antigens and antibodies, and in similar fashion, between receptors and ligands. A novel approach to the search for antigenic sequences which requires no previous knowledge of protein sequence or native epitopes involves the synthesis of "peptide libraries"; screening these libraries with monoclonal antibodies raised against a native protein facilitates the discovery of not only linear epitopes but also "mimotopes", a concept introduced to describe discontinuous epitopes which mimic the essential binding properties of a native epitope (Geysen *et al.*, 1986). Both epitope mapping and library screening can be employed to identify candidates for the preparation of synthetic peptide vaccines, which should overcome toxicity problems of conventional whole cell vaccines and more easily incorporate antigenic variability.

These new approaches have prompted an escalating demand for synthetic peptides. While numerous automated synthesizers for both batchwise and continuous flow operation at various production scales have been available commercially since the introduction of the first instrument in the 1960s (Merrifield *et al.*, 1966), they have been restricted to single synthesis or a few simultaneous syntheses in series. This traditional technology is time-consuming and expensive when applied to analogue series, and totally impractical in the case of synthetic libraries where large numbers of peptides are involved - for example, a library of all possible hexapeptides requires the synthesis of 20^6 (i.e. 64,000,000) peptides! In response to the growing demand, a number of different concepts for multiple peptide synthesis (MPS) have been developed since the mid 1980s, yet various limitations have prevented commercialization in most cases. Available technology may be expensive or require a certain level of expertise which may pose a barrier to the novice. Peptide products may be provided in unsuitable format or insufficient yield for routine use (designed primarily for ease in immunological testing rather than for aqueous solution studies). In addition, attention has focussed on multiple peptide synthesis while neglecting the equally important areas of multiple cleavage and multiple purification. Peptide availability has once again become an issue, especially for those lacking experience in the field of peptide synthesis.

This project was designed to address the need for simple, reliable and inexpensive multiple peptide synthesis, cleavage, and purification, thereby ensuring the availability of synthetic peptides to any investigator in any field who perceives a need for them and wishes to pursue their use. The project has been divided into two stages: the first concerned with the development of instrumentation, methodology, and applications for a multiple peptide synthesizer capable of multiple cleavage, and the second devoted to the development of a complementary multiple peptide purification system.

Chapter 2 PEPTIDE SYNTHESIS BACKGROUND

2.1 Solid-Phase Peptide Synthesis

Current peptide synthesis methodologies are based on solid-phase peptide synthesis (SPPS), an approach conceived and developed by R. B. Merrifield to provide an alternative to classical solution-phase synthesis which requires reaction conditions and purification procedures to be optimized for each synthesis intermediate (Merrifield, 1963). The introduction of a covalent attachment step to anchor the nascent peptide at its C-terminal to an insoluble polymeric support or resin allows excess reagents to be used to drive reactions to completion, and ensures soluble byproducts and unused reagents can be removed easily by filtration. Solid-phase synthesis proceeds at ambient temperature with high fidelity and yield using chemistries (protection schemes and reagents) proven in solution-phase synthesis, while avoiding the manipulation losses associated with the latter. A number of comprehensive reviews of SPPS have been written, including Erickson and Merrifield (1976), Barany and Merrifield (1979), Stewart and Young (1984), Merrifield (1986), Barany *et al.* (1987), Atherton and Sheppard (1989), Fields and Noble (1990), Fields *et al.* (1992), and Merrifield (1995).

As outlined in **Figure 1**, SPPS is based on sequential addition of α-amino and sidechain protected amino acid residues to the anchored peptide. "Temporary" blocking of the α-amino group during residue addition is provided by a labile protecting group and ensures peptide growth in the C-terminal to N-terminal direction, while "permanent" sidechain protecting groups prevent side reactions of trifunctional residues during peptide elongation. Each addition cycle consists of deprotection, neutralization (depending on protection strategy), and coupling steps. Deprotection involves removal of the peptide's N^{α}-protecting group, and the resulting charged peptide intermediate may require neutralization before residue addition can proceed. Peptide bond formation requires an activated carboxyl function on the incoming amino acid residue, and this is provided in the coupling step as a pre-activated derivative or is generated by an *in situ* coupling reagent. Once peptide elongation is complete, the final N^{α}-protecting group is removed before cleaving the peptide from the resin under conditions which are minimally destructive towards sensitive residues in its sequence. Sidechain protecting groups, which remain in place during peptide elongation, are chosen normally so as to be released simultaneously with peptide cleavage from the resin, but they may be retained to provide protected fragments for further condensation or to undergo specialized deprotection procedures later in solution. Synthesis of homogeneous peptides requires that deprotection and coupling proceed to completion and both the peptide-resin anchorage and sidechain protection remain completely stable through each addition cycle.

The most widely used solid supports in SPPS are gel-type resins, i.e. inert, highly solvated and flexible polymer networks. Ideal for assembling relatively large molecules such as peptides, these matrices are composed of polystyrene, polyacrylamide, polyethylene glycol (PEG)-grafted polystyrene, or PEG-based composites, with evenly distributed functional groups acting as anchoring sites for peptide synthesis. Hydrophobic polystyrene resins crosslinked with 1% divinylbenzene (DVB) were the original SPPS supports developed by Merrifield and continue to be used today; average bead sizes of 10-200 μm are available with chloromethyl, hydroxymethyl, or aminomethyl functional groups in typical loadings (i.e. functionalization levels) of 0.2-1.0 mmol/g. Polystyrene resin swells best in non-polar solvents such as dichloromethane (DCM); unfortunately, these solvents do not solvate peptide chains very well due to the hydrophilic nature of the peptide backbone, and resin swelling may change dramatically over the synthesis, making these supports unsuitable for continuous flow operation. Peptide synthesis efficiency can be improved with the use of a solvent such as dimethylformamide (DMF) which not only swells the resin but also solvates peptide chains.

Polyacrylamide resins were conceived as a means to overcome the problem of inadequate peptide solvation leading to premature peptide termination, by providing a polarity comparable to the peptide chain being synthesized (Atherton *et al.*, 1978). This hydrophilic alternative to polystyrene resin consists of poly-*N,N*-dimethylacrylamide crosslinked with 5% bis-*N,N'*-acryloylethylenediamine and functionalized with *N*-acryloylsarcosine methyl ester functional groups. In contrast with polystyrene resin, polyacrylamide resin swells best in polar solvents such as DMF, but the improved flexibility is accompanied by greater fragility and insufficient mechanical stability to withstand continuous flow conditions. The problem of resin stability under these conditions has been solved by the development of low density, high permeability rigid matrices to support gel-type resins. Kieselguhr, an inert and porous material prepared from sediments, is one example of such a material, and is used as a matrix for dimethylacrylamide polymerization to overcome the problem of gel deformation (Atherton *et al.*, 1981); unfortunately, Kieselguhr is highly heterogeneous with a tendency to generate fines (causing reduced flow and back pressure build-up), and the supported resin suffers from low loadings (typically around 0.1 mmol/g). Macroporous polystyrene particles with a high degree of crosslinking (i.e. 50%) provide an alternative scaffold with improved flow properties, and polyacrylamide resin can be grafted by acryloylation to the matrix at aminomethyl functional groups (Small and Sherrington, 1989). These polyacrylamide-Polyhipe supported resins also permit higher loadings (0.3-1.0 mmol/g). Polystyrene properties have also been modified successfully for continuous flow operation. Grafting amphipathic PEG on to a polystyrene resin in a weight ratio of approximately 1:1 allows resin swelling in both non-polar and polar solvents, provides reasonably constant swelling throughout peptide synthesis, and ensures a more mechanically stable support suitable for use in both batch and continuous flow modes of operation (Hellerman *et al.*, 1983; Rapp *et al.*, 1989). The PEG chains also act as spacers, distancing the site of peptide synthesis from the resin backbone by 7 to 10 (or more) ethylene glycol residues. One drawback is a lower loading than obtained with polystyrene resins. New resins have also been obtained by polymerizing functionalized PEG with a small amount of polystyrene or polyacrylamide, or with polypropylene glycol (PPG) to form a PEG/PPG network; the result is a highly crosslinked polymer with long PEG links (Meldal, 1992; Renil and Meldal, 1995 and 1996).

Within the last decade there has been considerable experimentation with alternative solid supports for SPPS. Surface-type supports require regular topology and a large surface area to provide an appropriate level of functional groups as sites for peptide attachment and to allow unhindered reagent access. Materials which have been tested include controlled pore glass (Büttner *et al.*, 1988), cellulose fibres (Frank and Döring, 1988), polyethylene filters (Büttner *et al.*, 1994), and sintered polyethylene particles (Cook and Hudson, 1996). While some of these materials show promise, none are available commercially. Surface-type supports can be extended by grafting linear polymers (normally polystyrene) onto a rigid surface such as non-crosslinked polyethylene to form film or brush polymers (Berg *et al.*, 1989), but the linear polymers are restricted to one-dimensional swelling and this may hinder the synthesis of long peptides.

The choice of protection chemistry is generally the key factor in the success of a peptide synthesis. The "temporary" N^{α}-protecting group must be sufficiently labile to allow quantitative removal before each residue addition, while "permanent" sidechain protecting groups must be capable of withstanding repeated N$^{\alpha}$-deprotection yet be removed under conditions causing minimal effects on the completed peptide chain. Two major protection strategies exist in SPPS. The approach developed in the classic Merrifield synthesis employs a common chemical mechanism for the removal of both classes of protection, relying on kinetic "fine-tuning" to ensure sufficiently large differences in reactivity between the "temporary" and "permanent" groups. Boc strategy **(Figure 2)** relies on graduated acid lability of the N$^{\alpha}$ and sidechain protecting groups: the *t*-butyloxycarbonyl (Boc) group of McKay and Albertson (1957) is used for N$^{\alpha}$-protection and can be removed by moderately strong trifluoroacetic acid (TFA), while the benzyl-derived groups (ether, ester, and urethane derivatives) commonly selected for sidechain protection are removed simultaneously with peptide cleavage by a strong acid such as hydrogen fluoride (HF), trifluoromethane-sulfonic acid (TFMSA), or trimethylsilyltrifluoroacetate (TMSOTf). The Boc group is released as isobutylene and carbon dioxide, leaving behind the acid salt of the peptide-resin which must be neutralized by a tertiary amine such as triethylamine (TEA) or *N,N* - diisopropylethylamine (DIEA) to generate the free amine for coupling **(Figure 3)**. Incomplete neutralization can lead to activation of the TFA carboxyl function during the subsequent coupling step and premature peptide termination through trifluoroacetylation of the deprotected N-terminal.

Concern over the effect of repetitive acidolysis on the peptide-resin link (especially with increasing peptide length) and the possibility of acid-catalyzed side reactions has led to the development of an alternative orthogonal protection scheme, which relies on different mechanisms for the removal of the two classes of protecting groups. Since selectivity is based on differences in chemistry rather than reaction rate, the protecting groups can be removed in any order and in the presence of another class, which permits substantially milder conditions for removal and allows synthesis of protected fragments. By far the most common example of this strategy employs the 9-fluorenylmethyloxycarbonyl (Fmoc) group of Carpino and Han (1972) for N^{α}-protection, and this group is removed by mild base treatment using a secondary amine such as piperidine; *t*-butyl-derived groups are commonly selected for sidechain protection, and are removed simultaneously with acidolytic cleavage of peptide from the resin by TFA **(Figure 4)**. The Fmoc group is released as carbon dioxide and a highly reactive dibenzofulvene intermediate which is rapidly trapped by the secondary amine; a neutralization step is not required since the base-catalyzed β-elimination mechanism proceeds through a carbanion intermediate **(Figure 5)**. This mild procedure is the method of choice for the synthesis of most modified peptides, including glycosylated, phosphorylated, and sulfated peptides. Fmoc protection strategy is employed in continuous flow operation due to the capability for real-time spectrophotometric monitoring of deprotection: the release of the Fmoc group can be followed at 300-320 nm to determine the progress of the reaction.

Sidechain protection for trifunctional amino acids must be compatible with N^{α}-protection strategy, and is designed to minimize potential side reactions during coupling and the final deprotection/cleavage step. Protection is essential for the sidechains of aspartate (Asp), glutamate (Glu), lysine (Lys) and cysteine (Cys) residues, and may be desirable for other residues depending on peptide length, sequence, and chosen activation chemistry. A number of common side reactions are presented in **Figure 6**, and some recommended sidechain protecting groups for Boc and Fmoc strategies are listed in **Table 1**. The sidechain carboxyl functions of Asp and Glu are routinely protected as benzyl (OBzl) and *t*-butyl (OtBu) esters in Boc and Fmoc strategies respectively. During cleavage, Asp is susceptible to intramolecular cyclization through elimination of the sidechain protecting group and formation of an aspartimide, which then partitions to the desired α-peptide and a byproduct in which the peptide chain grows from the β-carboxyl function. Glu may also undergo cyclization, with subsequent rearrangement to form the γ-glutamyl peptide. Depending on sequence, the α-to-β rearrangement may occasionally pose a serious problem, which may be minimized by using a sterically hindered ester for protection of the β-carboxyl function, as provided by the 1-adamantyl (O-1-Ada) group in Fmoc strategy and either the 2-adamantyl (O-2-Ada) or cyclohexyl (OcHx) group in Boc strategy. The related uncharged amino acids asparagine (Asn) and glutamine (Gln) may be incorporated without sidechain protection, although there is the possibility of carboxamide sidechain dehydration and nitrile formation upon activation and Gln may also undergo acid-catalyzed intramolecular cyclization in Boc chemistry to form pyroglutamate during chain elongation (bringing about premature chain termination). Sidechain protecting groups such as 9-xanthenyl (Xan) in Boc strategy and 2,4,6-trimethoxybenzyl (Tmob) or trityl (Trt) in Fmoc strategy can minimize these problems, but may introduce others: the Xan group is not completely stable to N^{α}-deprotection, the Trt group may cause steric hindrance of chain elongation, and all three groups may modify adjacent Trp residues during cleavage.

The ε-amino function of Lys is best protected by the 2-chlorobenzyloxycarbonyl (2-ClZ) group in Boc strategy and by the Boc group in Fmoc strategy, while the guanidino sidechain of arginine (Arg) can be protected by benzenesulfonyl derivatives such as 4-toluenesulfonyl (Tos) or mesitylene-2-sulfonyl (Mts) in Boc strategy and by 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) or the more acid-labile 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) in Fmoc strategy. Most Arg derivatives are susceptible to δ-lactam formation during activation, resulting in peptide chain termination if the δ-lactam transfers the protected guanidino group to a free amino group; couplings are commonly performed in the presence of HOBt (1-hydroxybenzotriazole) to prevent this intramolecular aminolysis. Histidine (His) is very susceptible to base-catalyzed racemization after proton abstraction from the optically active α-carbon by the imidazole π-nitrogen, but racemization can be suppressed by N^{π}-blocking or by reducing the basicity of the imidazole ring through N^{τ}-blocking. The former is accomplished by use of the benzyloxymethyl (Bom) group in Boc strategy and the *t*-butoxymethyl (Bum) group in Fmoc strategy, although final deprotection releases formaldyhyde which in turn can modify other susceptible sidechains. The alternative N^{τ}-blocking may also pose difficulties: in Boc strategy, the Tos group must be accompanied by appropriate selection of activation chemistry to avoid premature loss of the protecting group, while the 2,4-dinitrophenyl (Dnp) group requires a separate final deprotection using thiophenol before cleavage. In Fmoc strategy, this form of blocking can be achieved with the Boc group (although it is only reasonably stable to repetitive base-catalyzed deprotection), or more securely with the Trt group.

Protection of the sulfhydryl function of the Cys sidechain is the most challenging. Many protecting groups are available for Cys, including those which are retained through the cleavage step for removal after peptide purification (to allow formation of disulfide bridges); the latter require special reagents and procedures for removal: for example, the acid- and base-stable acetamidomethyl (Acm) group is removed by mercuric acetate followed by treatment with H_2S or excess mercaptan to free the thiol, or the protected residue is converted directly to a disulfide by treatment with I_2 or $Tl(Tfa)_3$ (thallium trifluoroacetate). Of the groups compatible with Boc strategy, the 4-methylbenzyl (Meb) group is optimized for removal by strong acid, and the protected residue may also be directly converted to the oxidized form (i.e. cystine) using $Tl(Tfa)_3$, while Fmoc-compatible Tmob and Trt groups are removed by TFA in the presence of scavengers to prevent Cys realkylation by the resulting stable carbonium ions. No protection is necessary for the sidechains of methionine (Met) and tryptophan (Trp) using Fmoc strategy, but there may be occasion for protection in Boc strategy, in which case the sulfoxide (O) and formyl (CHO) groups are employed for Met and Trp respectively. The sidechain hydroxyl functions of serine (Ser), threonine (Thr), and typrosine (Tyr) can be protected as benzyl (Bzl) and *t*-butyl (tBu) ethers in Boc and Fmoc strategy respectively, but during cleavage in strong acid the Bzl group on the Tyr phenol can migrate to the 3-position of the ring, making 2,6-dichlorobenzyl (2,6-Cl_2Bzl) or 2-bromobenzyloxycarbonyl (2-BrZ) preferable for Tyr protection in Boc strategy. Deprotected Ser and Thr residues may experience N-to-O acyl shifts (i.e. intramolecular migration of the peptide chain from the α-amino to the hydroxyl function) during strong acid cleavage of the peptide from the resin, but this is a very slow and reversible process.

The nature of the peptide-resin link anchoring the nascent peptide C-terminal residue via its α-carboxyl group is also affected by the choice of protection strategy. In classic Merrifield synthesis based on graduated acidolysis, the first amino acid residue is attached through a *p*-alkylbenzyl ester link to a chloromethyl- or hydroxymethyl-functionalized polystyrene resin, and this ester link is cleaved by strong acid to yield the free peptide acid **(Figure 7)**. Unfortunately, the link is slightly labile to TFA removal of the Boc group during deprotection. The benzyl ester link can undergo different reactions to yield a variety of C-terminal end groups: ammonolysis (for a peptide amide), aminolysis (for a substituted peptide amide), reduction (for a peptide alcohol), transesterification (for a peptide ester), and hydrazinolysis (for a peptide hydrazide, which can undergo further azide couplings in solution). However, these alternate cleavage procedures are not suitable for peptides containing Asp or Glu residues, since their sidechain carboxyl functions will be modified as well. This has led to the development of resins which anchor the peptide directly through the desired C-terminal functionality. Most important of these are the amino-functionalized resins, which anchor the peptide through an amide bond and are designed to yield a C-terminal amide on cleavage by transferring the amino function from the support to the released peptide **(Figure 8)**. Aminomethyl-functionalized resins are not suitable for this purpose, since the resulting peptide-resin link is completely stable to acid cleavage; however, modifications such as the addition of a phenyl ring to form benzhydrylamine (BHA) resin permits cleavage by strong acid (although yield may be affected by the nature of the C-terminal residue), and an additional methyl substitution to form 4-methylbenzhydrylamine (MBHA) resin provides a more appropriately labile peptide-resin link by virtue of the electron-donating ability of the methyl group. Other resin functional groups are available to release a variety of C-terminal modified peptides by the standard acidolysis of Boc and Fmoc strategies or the use of other cleavage agents. Examples of functionalized resins and the resulting C-terminal end groups produced on cleavage are presented in **Table 2**.

The development of bifunctional spacers known as linkers or handles provides a permanent attachment to the functionalized resin at one end of the handle (usually by means of an amide bond, although other links are possible) and a temporary attachment to the growing peptide at the other end **(Figure 9)**. Advantages of employing a handle include potential use of any previously funtionalized resin for synthesis, greater control over substitution levels, more versatile chemistry in the form of cleavage mechanism (i.e. acidolysis, reduction, photolysis, etc.), C-terminal end group, and compatible N^{α}- and sidechain protection, and minimization of side reactions. Cyclic peptides can be synthesized using handles which attach to a sidechain or to an amide N in the peptide backbone. A new element is the incorporation of an internal reference amino acid (IRAA) such as norleucine between the handle and the resin functional group to provide a means of monitoring yields. Generally, the handle is attached to the resin before addition of the first amino acid residue, but a pre-formed version exists in which the handle is linked to the initial residue, circumventing the possibility of direct peptide anchorage to resin functional groups. A selection of handles is presented in **Table 3**. The desire to develop a more acid-stable peptide-resin link than that provided by the classic Merrifield resin led to the development of the first PAM (4-hydroxymethylphenyl acetic acid) handle, which increases link stability by a factor of 100 through the addition of the electron-withdrawing acetamido group. Much of the subsequent effort has been directed at diversification by decreasing the acid stability of a handle to allow faster cleavage and/or use of a milder cleavage agent such as TFA in Fmoc strategy. This involves fine-tuning the structural and electronic features of the handle, and a common theme is the addition of electron-donating substituents, exemplified in the progressive acid lability of the handle series PAM < HMPA < HMPB < HAL by the corresponding addition of *p*-alkyl, *p*-alkoxy, *p*- and *o*-alkoxy, and tris-alkoxy substituents. The most common handles employed in Fmoc strategy are the HMPA handle for peptide acids and the Rink handle for peptide amides.

Once the nascent peptide is anchored to the chosen support, peptide bond formation with an incoming N^{α}-protected amino acid can only proceed with high coupling efficiency if the amino acid C^{α}-carboxyl group is activated. The best methods of activation generally involve conversion of the amino acid to a derivative with a good leaving group, but this tends to increase the risk of racemization around the α-carbon through formation of an oxazolone **(Figure 10)**. Fortunately, the presence of an alkoxy group in urethane N^{α}-protecting groups such as Boc and Fmoc reduces the tendency to form an oxazolone, and decreases the tendency of such an oxazolone to racemize. Coupling methods can be divided into two major classes: coupling via pre-formed activated amino acid derivatives, and coupling via *in situ* activation of amino acids. Advantages of using the former include the high reactivity of the isolated derivatives and the simple byproducts formed. The most common pre-formed activated species are acid halides and active esters (**Figure 11**). Amino acid chlorides have limited use since they can only be prepared and successfully stored using Fmoc-amino acids without *t*-butyl sidechain protection; amino acid fluorides are more widely used due to compatibility with *t*-butyl-based sidechain protecting groups, high solubility in organic solvents, greater stability, reduced risk of racemization, and the ability to couple successfully multiple sterically hindered residues. Common pre-formed active esters include pentafluorophenyl (OPfp) and 1-oxo-2-hydroxy-dihydrobenzotriazine (ODhbt) esters; 1-hydroxybenzotriazole (OBt) and the recently introduced 7-aza-1-hydroxybenzotriazole (OAt) esters are less stable and are not normally isolated. OPfp esters react more slowly but their use allows the incorporation of some amino acids (Asn, Fmoc-Ser, and Fmoc-Tyr) without sidechain protection, and the addition of HOBt or HOAt will speed up coupling; ODhbt esters react rapidly, but ester stability declines with prolonged storage.

Common coupling reagents which instead rely on *in situ* activation of amino acids include carbodiimides and phosphonium and aminium salts. *N,N'*-dicyclohexylcarbodiimide (DCC) is the most widely used of the carbodiimides in Boc strategy, although its byproduct *N,N'*-dicyclohexylurea (DCU) is insoluble in DCM and must be removed by filtration; other carbodiimides generate urea byproducts which are more soluble in DCM. *N,N'*-diisopropylcarbodiimide (DIPCDI) is used in Fmoc strategy due to the solubility of its urea byproduct in DMF. Carbodiimide activation proceeds through an O-acylisourea intermediate formed by interaction of the protected amino acid with the carbodiimide in a 1:1 ratio; the use of a 2:1 ratio allows the intermediate to react with the second equivalent of amino acid to produce the corresponding symmetric anhydride, while activation in the presence of HOBt produces the active OBt ester (**Figure 12**). All three activated species are capable of forming the peptide bond, but the symmetric anhydride is the least efficient due to the coupling of only one of the two equivalents of amino acid. The presence of HOBt during generation of the active ester reduces the risk of racemization and prevents carboxamide dehydration during the coupling of Asn and Gln without sidechain protection. A modification for Boc strategy involves DCC-based preactivation of protected amino acids in DCM to form symmetric anhydrides; DCU is removed by filtration, and solvent exchange allows optimal coupling to proceed in DMF.

Other *in situ* activating reagents are phosphonium salts based on either HOBt or the recently introduced HOAt; the former includes (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) and (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), while HOAt-based examples include (7-azabenzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (AOP) and (7-azabenzotriazol-1-yloxy)tris(pyrrolidino)-phosphonium hexafluorophosphate (PyAOP) as depicted in **Figure 13**. The reactive species formed in the presence of an equivalent of a tertiary base such as DIEA or *N*-methylmorpholine (NMM) are the OBt and OAt esters **(Figure 14)**. Aminium analogues of phosphonium salts include *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethan-aminium hexafluorophosphate *N*-oxide (HATU), and 1-(1-pyrrol-idinyl-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene)pyrrolidinium hexafluorophosphate *N*-oxide (HAPyU), and the tetrafluoroborate TBTU and TATU equivalents **(Figure 15)**.

The removal of sidechain protecting groups and release of the completed peptide are normally performed simultaneously in the final deprotection/cleavage step, and the reagents and procedures used depend once again on the protection strategy, although acidolysis is the procedure of choice **(Figure 16)**. Acidolytic cleavage of sidechain protecting groups generates reactive carbonium and acylium ions which can attack residues susceptible to alkylation: for example, benzyl and *t*-butyl carbonium ions readily alkylate Cys, Met, Trp and Tyr residues. However, these side reactions can be prevented by the addition of suitable nucleophilic scavengers to the cleavage reaction mixture. The most popular strong acid reagent in Boc strategy is anhydrous liquid HF, which appears to be the most versatile and least harmful cleavage reagent for a wide variety of peptides. Both time and temperature are important factors in minimizing side reactions, and HF cleavage is generally performed at 0° - 5° C for 45 - 60 minutes. A preliminary cleavage at a lower temperature may be performed to assist the removal of sidechain protecting groups, but this can be very slow and impractical when residues such as Lys (2-ClZ) and Arg (Tos) are present. Anisole is one of the most widely used scavengers, and other possibilities include DMS (dimethyl sulfide), *m*- or *p*-cresol, resorcinol, thioanisole, EDT (1,2-dithioethane), and ethyl methyl sulfide, or mixtures of several scavengers; thioanisole should not be used with Trp-containing peptides since thioanisole cation adducts can alkylate the indole ring nitrogen of Trp. Many of the side reactions observed in "difficult" cleavages can be circumvented by the low-high procedure of Tam *et al*. (1983), which employs an equimolar mixture of HF and DMS (1:3 by volume) to change the cleavage mechanism from S_N1 (in which carbonium and acylium ions are produced) to S_N2 (in which DMS attacks the protonated intermediate before the carbonium ion can form). This procedure prevents alkylation of Tyr by Bzl and 2,6-Cl_2Bzl cations, acylation of scavengers by Glu side chains, and formation of succinimide peptides from Asp-Gly sequences, and reduces Met sulfoxide residues to Met. The procedure alone may not be sufficient to cleave peptides from MBHA resins if the C-terminal residue is sterically hindered, nor will it remove all sidechain protecting groups, but these problems can be remedied by following the procedure with a standard HF cleavage.

In Fmoc strategy, the acidolytic cleavage agent is normally TFA, and cleavage is performed at room temperature. Under certain circumstances, TFA may cleave at both the peptide-handle link and at the point of attachment of the handle to the resin, giving rise to handle carbonium ions which can alkylate Trp, and TFA itself may react with *t*-butyl cations to form tBu trifluoroacetate. Highly reactive carbonium ions and sulfonyl species generated on cleavage of the sidechain protecting groups can alkylate or sulfonate susceptible amino acid residues, especially Met, Trp, and Tyr. Nucleophilic scavengers are required to prevent irreversible modifications to vulnerable residues of the peptide, and may even assist in the removal of stubborn sidechain protecting groups. The scavenger EDT traps tBu, tBu trifluoroacetate, Pmc, and linker cations, preventing their attachment to Met, Trp, or Tyr residues and reattachment of Trt to Cys, and also prevents oxidation of Trp. Water quenches the reactivity of tBu and Pmc ions and inhibits the formation of a TFA adduct with EDT. Thioanisole prevents *t*-butylation and oxidation of Met and speeds up the removal of aryl sulfonyl protecting groups, and phenol assists in scavenging Pmc cations. As in Boc strategy, multiple scavengers are often required to quench all the reactive species originating from sidechain protecting groups and resin handles. Some examples of scavenger-protecting group adducts resulting from TFA cleavage are presented in **Figure 17**.

The final step in solid-phase peptide synthesis is the recovery of the crude peptide from solution. Post-cleavage work-up usually consists of cold ether precipitation of the crude peptide; peptide yield is improved by removing TFA from solution with a rotary evaporator prior to performing the precipitation. As an alternative, the cleavage mixture can be subjected to an ether extraction using a 50:50 water to ether solution, after which the peptide can be recovered from the aqueous layer by lyophilization.

Figure 1 **Outline of Solid-Phase Peptide Synthesis**

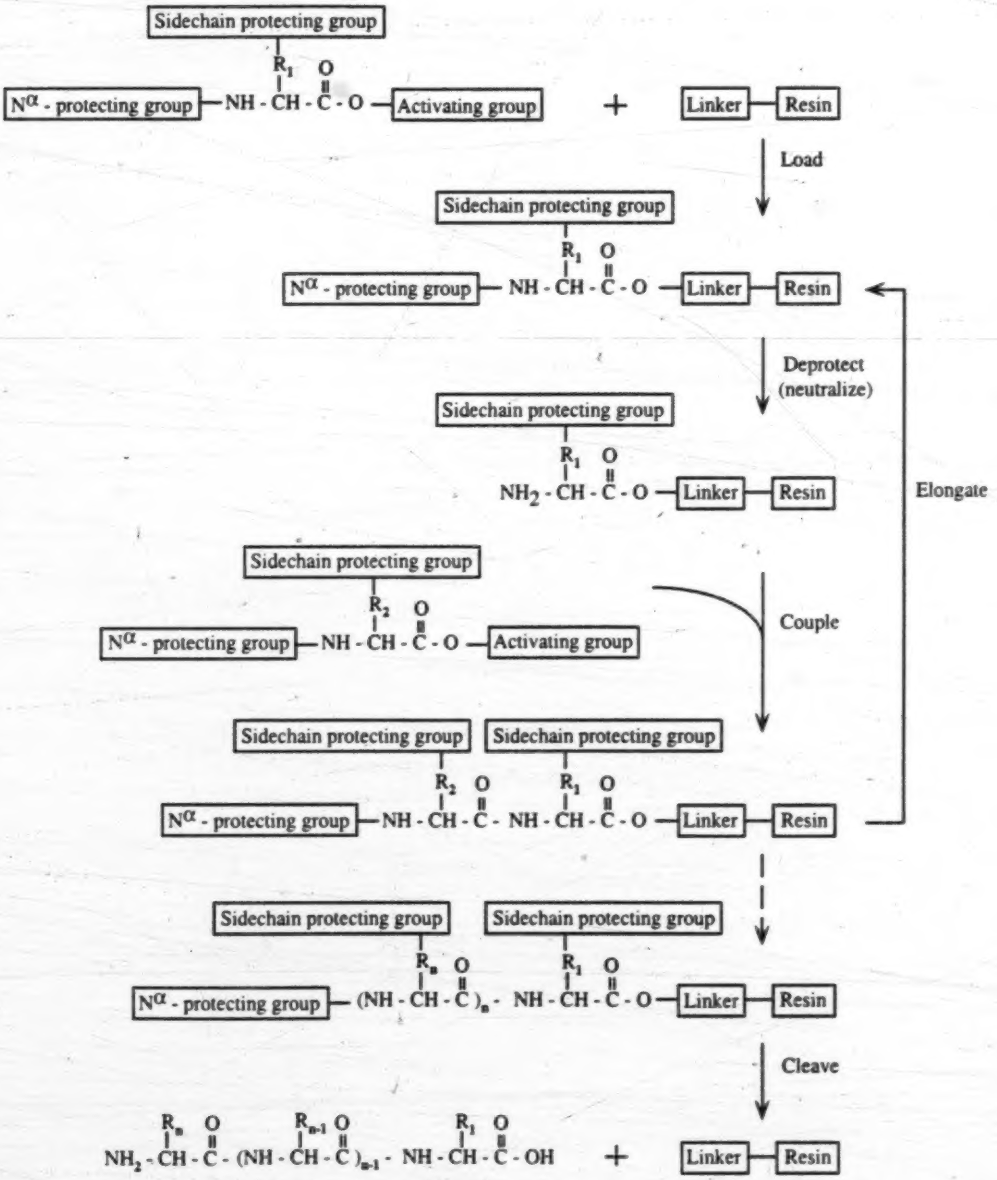
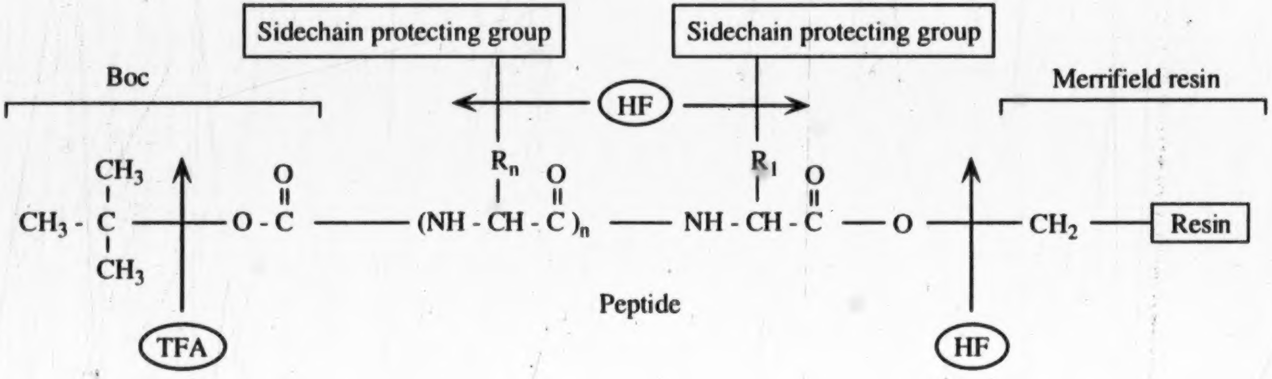
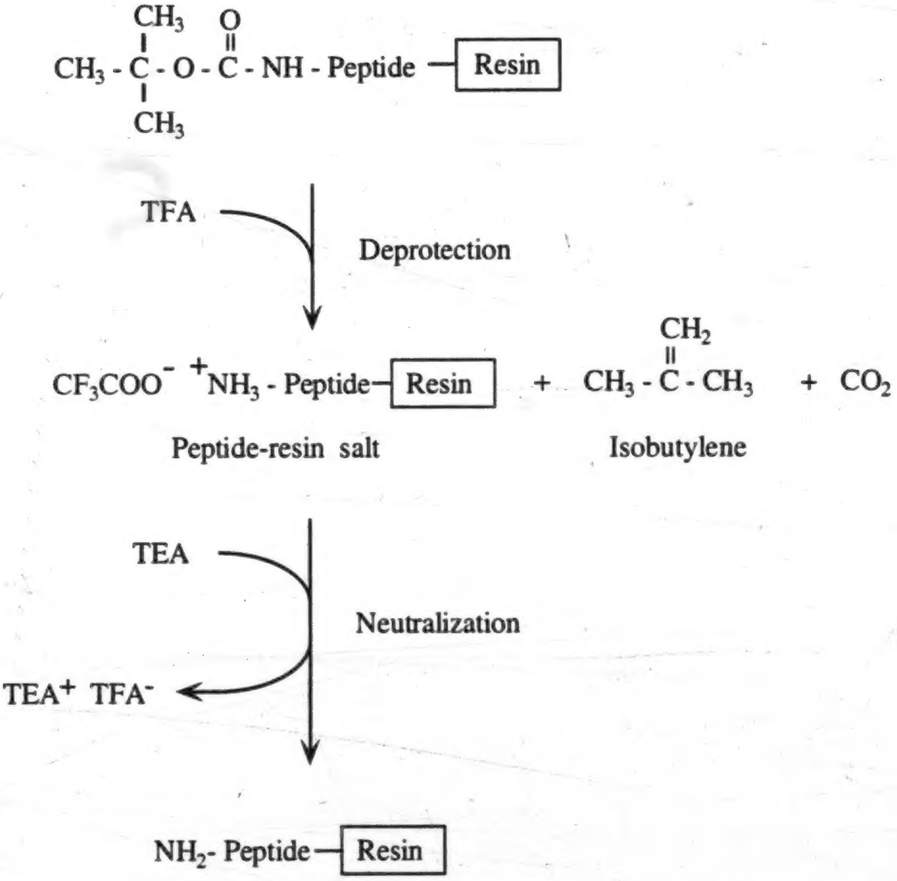

Sidechain protecting group
R_1
O
N^{α} - protecting group
NH - CH - C - O
Activating group
+
Linker
Resin
Load
Deprotect (neutralize)
NH_2 - CH - C - O
Elongate
Couple
R_2
R_n
(NH - CH - C)$_n$ -
Cleave
R_{n-1}
NH_2 - CH - C - (NH - CH - C)$_{n-1}$ - NH - CH - C - OH

Figure 2 Boc Strategy for SPPS


Sidechain protecting group
Sidechain protecting group
Boc
Merrifield resin
HF
R_n
R_1
$CH_3 - C(CH_3)_2 - O - C(=O) — (NH - CH - C)_n — NH - CH - C(=O) — O — CH_2 — Resin$
Peptide
TFA
HF

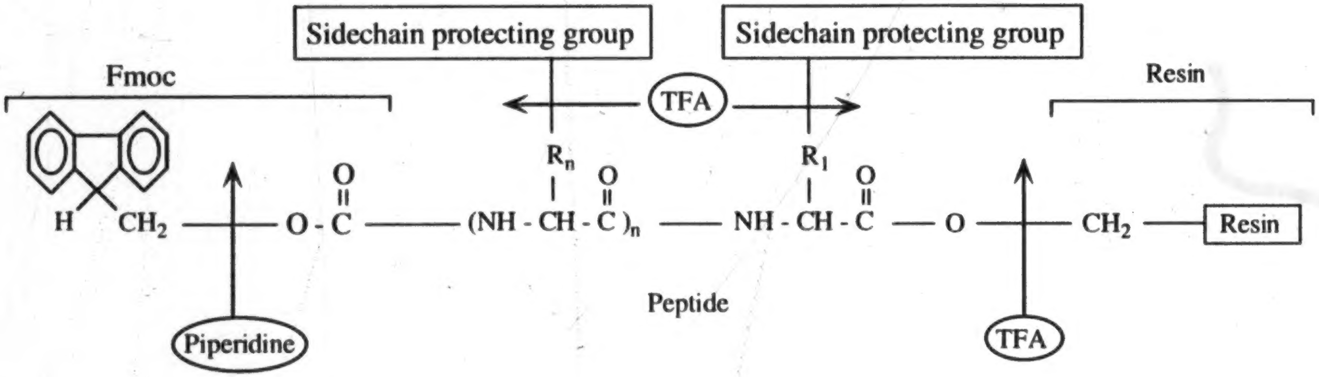
R = Sidechain of amino acid residue

Figure 3 Boc Deprotection



CH3
O
CH3 - C - O - C - NH - Peptide
Resin
CH3
TFA
Deprotection
CF3COO- +NH3 - Peptide
Resin
+
CH2
CH3 - C - CH3
+ CO2
Peptide-resin salt
Isobutylene
TEA
Neutralization
TEA+ TFA-
NH2- Peptide
Resin

Figure 4 Fmoc Strategy for SPPS



Sidechain protecting group
Sidechain protecting group
Fmoc
TFA
Resin
H
CH$_2$
O - C
O
(NH - CH - C)$_n$
R$_n$
NH - CH - C
R$_1$
O
CH$_2$
Resin
Peptide
Piperidine
TFA

R = Sidechain of amino acid residue

Figure 5 Fmoc Deprotection

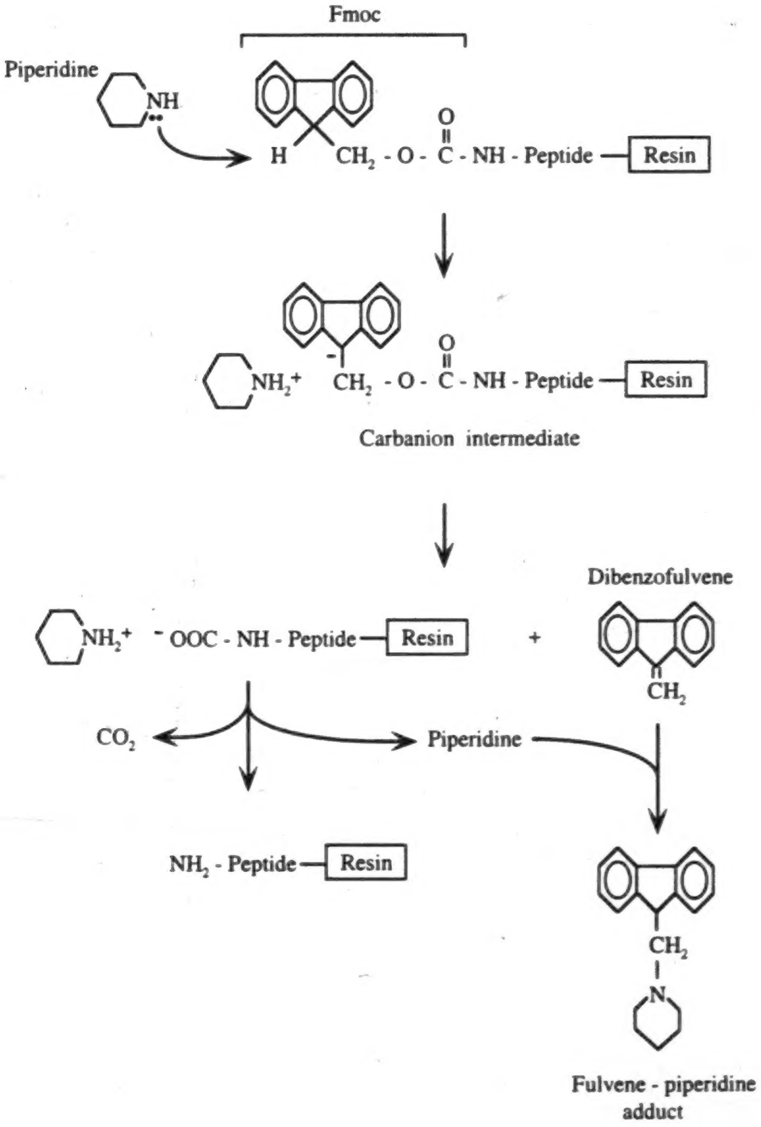
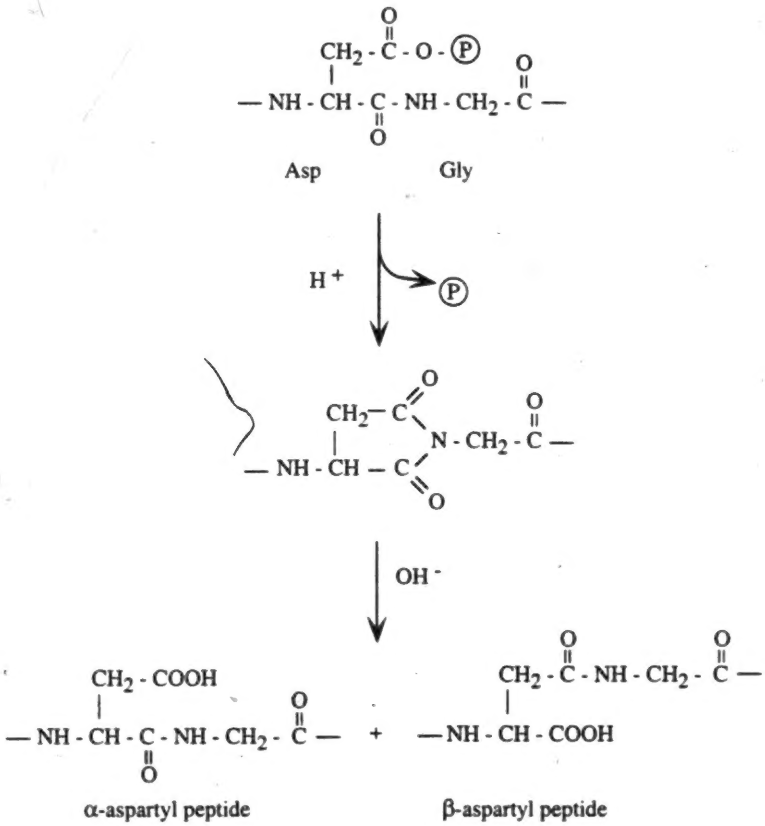

Fmoc
Piperidine
NH
H
CH_2 - O - C - NH - Peptide
O
Resin
NH_2^+
CH_2 - O - C - NH - Peptide
Resin
Carbanion intermediate
Dibenzofulvene
NH_2^+ $^-$OOC - NH - Peptide
Resin
+
CH_2
CO_2
Piperidine
NH_2 - Peptide
Resin
CH_2
N
Fulvene - piperidine adduct

Figure 6 Common Side Reactions in SPPS

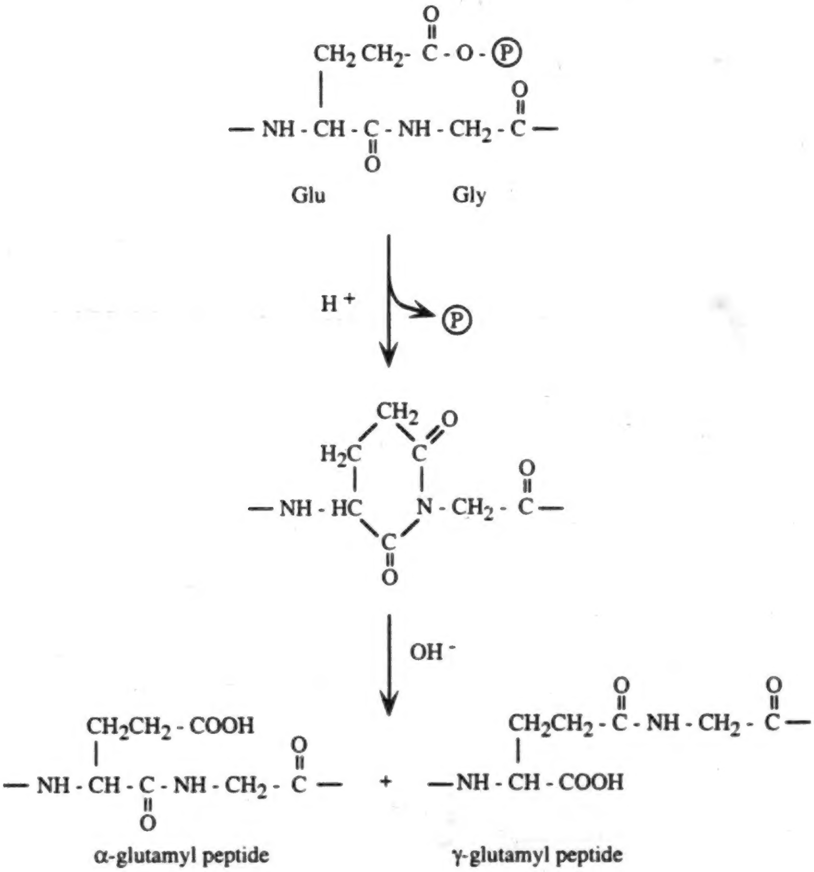
A. Formation of β-aspartyl peptide


Asp
Gly
H +
OH -
α-aspartyl peptide
β-aspartyl peptide

Ⓟ = sidechain protecting group

Figure 6 Common Side Reactions in SPPS (cont'd)

B. Formation of γ-glutamyl peptide


Glu
Gly
H +
OH -
α-glutamyl peptide
γ-glutamyl peptide

Ⓟ = sidechain protecting group

Figure 6 Common Side Reactions in SPPS (cont'd)

C. Dehydration of asparagine and glutamine

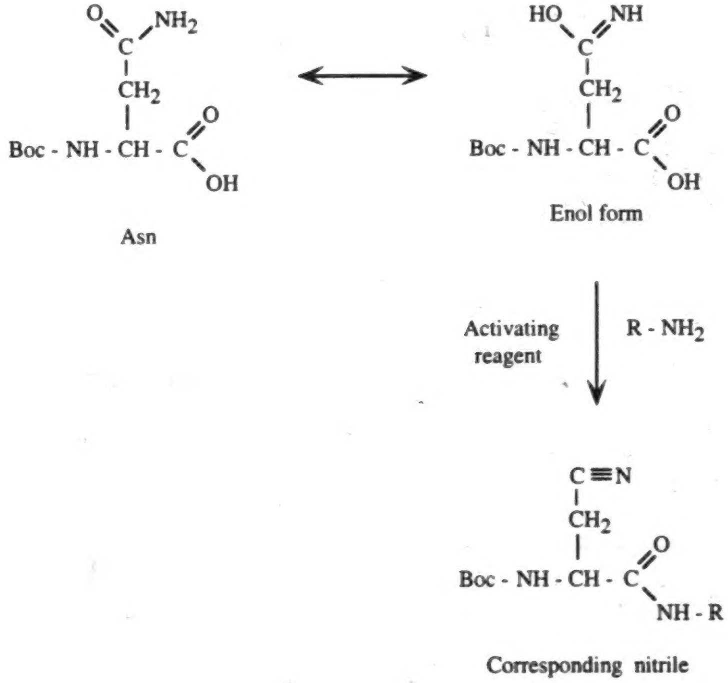
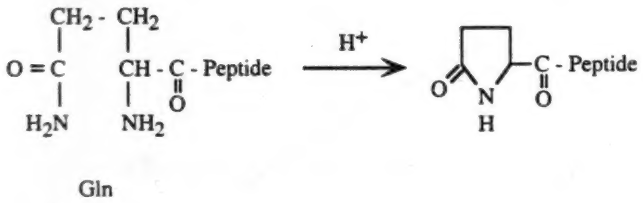
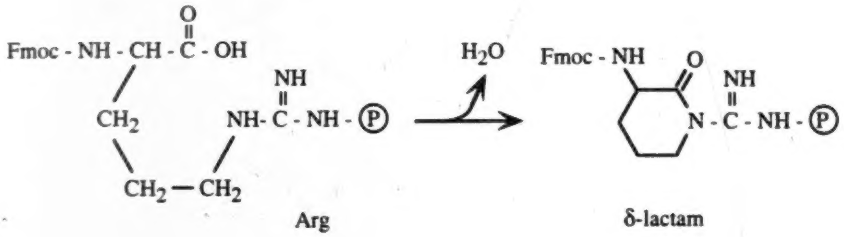

Asn
Enol form
Activating reagent
R - NH2
Corresponding nitrile

Figure 6 Common Side Reactions in SPPS (cont'd)

D. Chain Termination By Formation Of Pyroglutamate


H+
Peptide
Gln

E. Formation of δ-lactam from Arg


Fmoc
H2O
Arg
δ-lactam

F. N - to - O acyl shifts in Ser and Thr

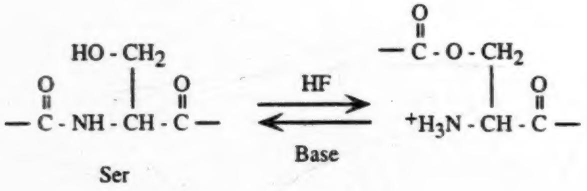


Table 1 Recommended Sidechain Protecting Groups

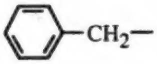
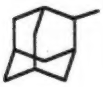
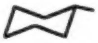
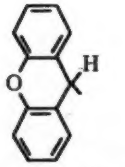
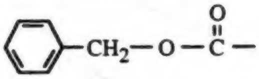
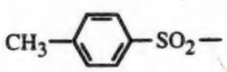
Protecting Group	Protected Amino Acid Derivative	Removal	References
Compatible with Boc chemistry:			
Benzyl 	Asp/Glu(OBzl) Ser/Thr/Tyr(Bzl)	Strong acid Strong acid	Barany and Merrifield (1979) Yajima *et al.* (1988)
2-Adamantyl 	Asp(O-2-Ada)	Strong acid	Okada and Iguchi (1988)
Cyclohexyl 	Asp(OcHx)	Strong acid	Tam and Merrifield (1987)
9-Xanthenyl 	Asn/Gln(Xan)	Strong acid	Dorman *et al.* (1972)
2-Chlorobenzyloxycarbonyl 	Lys(2-ClZ)	Strong acid	Erickson and Merrifield (1973b)
4-Toluenesulfonyl 	Arg(Tos) His(Tos)	HF Strong acid	Tam and Merrifield (1987) Stewart *et al.* (1972)

Table 1 Recommended Sidechain Protecting Groups (continued)

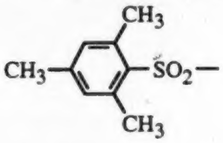
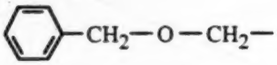
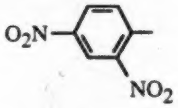
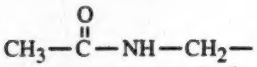
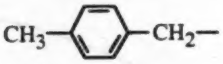
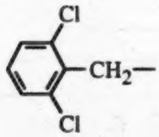
Protecting Group	Protected Amino Acid Derivative	Removal	References
Compatible with Boc chemistry:			
Mesitylene-2-sulfonyl 	Arg(Mts)	Strong acid	Yajima *et al.* (1978)
Benzyloxymethyl 	His(Bom)	Strong acid	Brown *et al.* (1982)
2,4-Dinitrophenyl 	His(Dnp)	Thiophenol	Chillemi and Merrifield (1969)
Acetomidomethyl  $CH_3-\overset{O}{\overset{\|}{C}}-NH-CH_2-$	Cys(Acm)	Hg(II) I_2 $Tl(Tfa)_3$	Veber *et al.* (1972)
4-Methylbenzyl 	Cys(Meb)	Strong acid $Tl(Tfa)_3$	Erickson and Merrifield (1973a)
Sulfoxide $-\overset{O}{\overset{\|}{S}}-$	Met(O)	low HF+DMS	Tam and Merrifield (1987)
Formyl - CHO	Trp(CHO)	low HF+DMS TFMSA	Bodanszky and Bodanszky (1984)
2,6-Dichlorobenzyl 	Tyr(2,6-Cl_2Bzl)	Strong acid	Erickson and Merrifield (1973a)

Table 1 Recommended Sidechain Protecting Groups (continued)

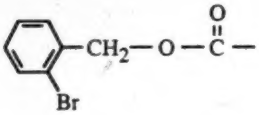
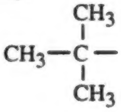
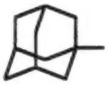
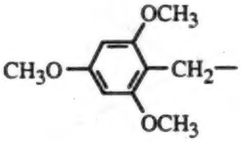
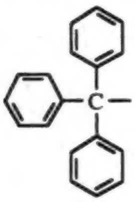
Protecting Group	Protected Amino Acid Derivative	Removal	References
Compatible with Boc chemistry:			
2-Bromobenzyloxycarbonyl 	Tyr(2-BrZ)	Strong acid	Yamashiro and Li (1973)
Compatible with Fmoc chemistry:			
t-Butyl  $(CH_3)_3C-$	Asp/Glu(OtBu)	TFA	Chang *et al.* (1980)
	Ser/Thr/Tyr(tBu)	TFA	Lajoie *et al.* (1990)
1-Adamantyl 	Asp(O-1-Ada)	TFA	Okada and Iguchi (1988)
2,4,6-Trimethoxybenzyl 	Asn/Gln(Tmob)	TFA	Weygand *et al.* (1968)
	Cys (Tmob)	TFA	Munson *et al.* (1992)
Triphenylmethyl (trityl) 	Asn/Gln(Trt)	TFA	Sieber and Riniker (1991)
	His(Trt)	TFA	Barlos *et al.* (1982)
	Cys(Trt)	TFA	Photaki *et al.* (1970)

Table 1 Recommended Sidechain Protecting Groups (continued)

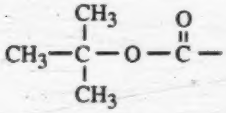
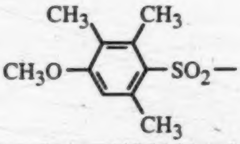
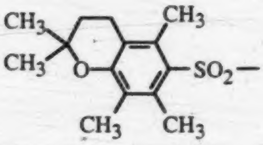
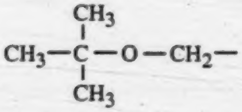
Protecting Group	Protected Amino Acid Derivative	Removal	References
Compatible with Fmoc chemistry:			
t-Butyloxycarbonyl	Lys(Boc)	TFA	Bodanszky and Bodanszky (1984)
 $CH_3-C(CH_3)_2-O-C(=O)-$	His(Boc)	TFA	Atherton and Sheppard (1989)
4-Methoxy-2,3,6-trimethylbenzenesulfonyl 	Arg(Mtr)	TFA	Fujino *et al.* (1981)
2,2,5,7,8-Pentamethylchroman-6-sulfonyl 	Arg(Pmc)	TFA	Ramage and Green (1987)
t-Butoxymethyl  $CH_3-C(CH_3)_2-O-CH_2-$	His(Bum)	TFA	Colombo *et al.* (1984)

Figure 7 Merrifield Peptide - Resin Link and Possible Cleavage Products

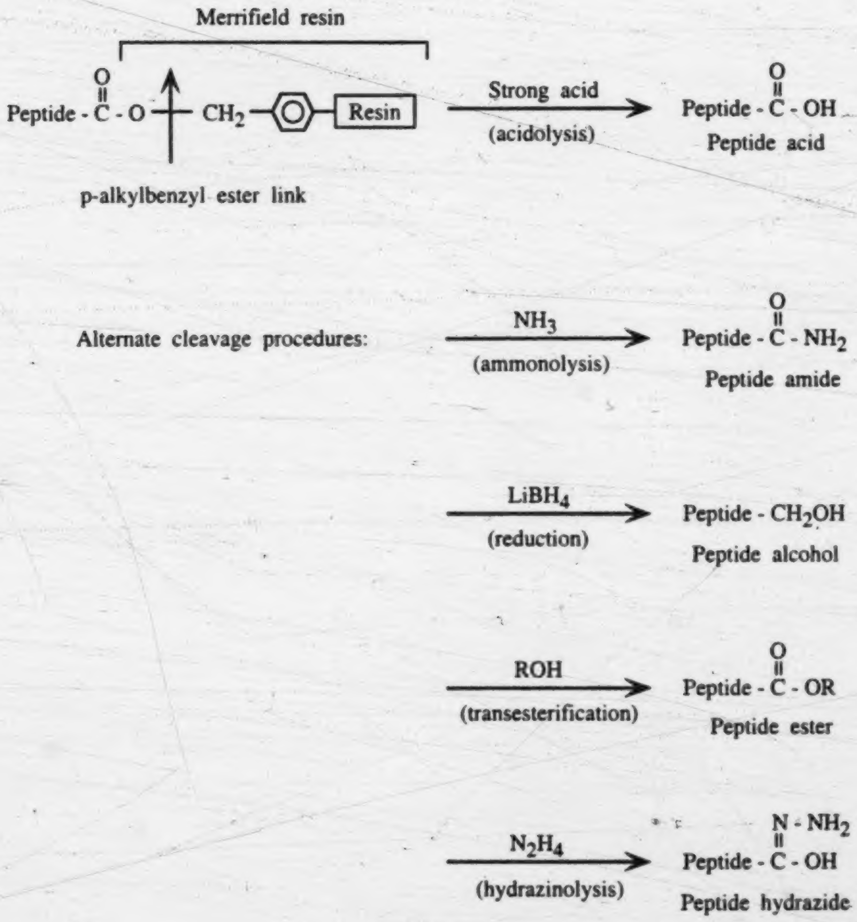
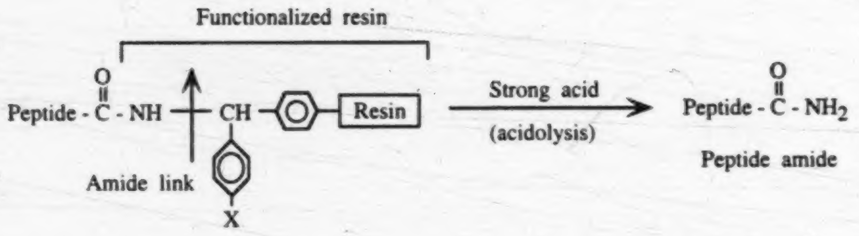

Merrifield resin
Peptide - C(=O) - O - CH2 - Resin
p-alkylbenzyl ester link
Strong acid
(acidolysis)
Peptide - C(=O) - OH
Peptide acid
Alternate cleavage procedures:
NH3
(ammonolysis)
Peptide - C(=O) - NH2
Peptide amide
LiBH4
(reduction)
Peptide - CH2OH
Peptide alcohol
ROH
(transesterification)
Peptide - C(=O) - OR
Peptide ester
N2H4
(hydrazinolysis)
Peptide - C(=N - NH2) - OH
Peptide hydrazide

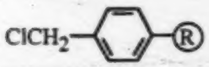
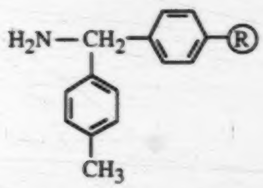
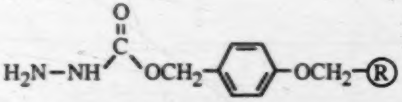
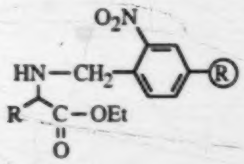
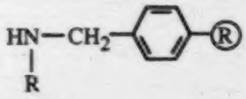
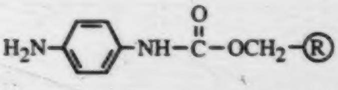
Figure 8 Peptide-Resin Link of BHA Type Amino-Functionalized Resins and Their Cleavage Products


Functionalized resin
O
Peptide - C - NH
CH
Resin
X
Amide link
Strong acid
(acidolysis)
O
Peptide - C - NH2
Peptide amide

Benzhydrylamine type resins :

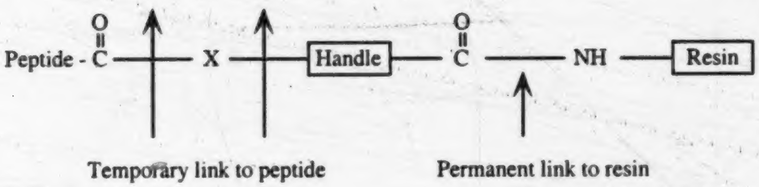
Benzylhydrylamine (BHA) resin	$X = H$
4 - methylbenzhydrylamine (MBHA) resin	$X = CH_3$

Table 2 Selected Functionalized Resins

Functionalized Resin	Resulting Peptide C-terminal	Cleavage agent	References
4-Chloromethyl (Merrifield) 	Acid	Strong acid	Gutte and Merrifield (1971)
4-Methylbenzhydrylamine (MBHA) 	Amide	Strong acid	Matsueda and Stewart (1981)
p-Alkoxybenzyloxycarbonyl-hydrazide 	Hydrazide	TFA	Wang (1973)
2-Nitrobenzylamine (N-terminal anchored amino ester substituted) 	Ester (R = amino acid sidechain)	hν (350 nm)	Renil and Pillai (1994)
N-alkylaminomethyl 	N-alkylamide (R = alkyl group)	HF	Rivier *et al.* (1977) Kornreich *et al.* (1985)
p-Aminoanilidocarbonyl-oxymethyl 	p-Nitroanilide	HF $NaBO_3$ / acetic acid	Burdick *et al.* (1993)

Resin polymer represented by Ⓡ on far right; resin functional groups drawn with the point of peptide attachment on far left.

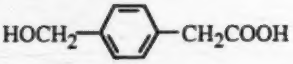
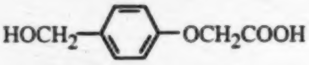
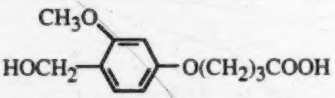
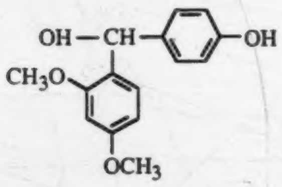
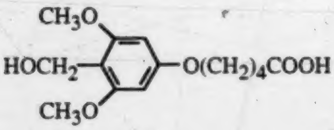
Figure 9 Anchoring of Peptide to Resin Using a Handle


O
Peptide - C
X
Handle
O
C
NH
Resin
Temporary link to peptide
Permanent link to resin

X may be derived from the handle or the first residue, and cleavage may occur on either side of X.

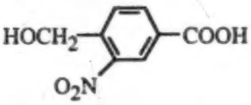
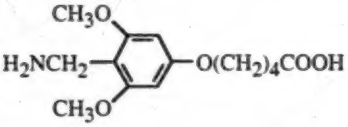
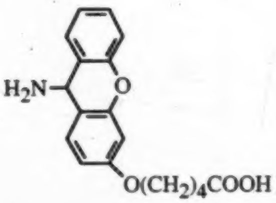
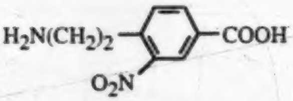
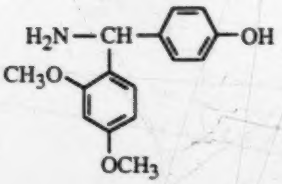
Possible cleavage products include:	Peptide acid (X=O) Peptide amide (X=NH) C-terminal modifed peptide

Table 3 Selected Resin Handles

Handle	Resulting C-terminal	Cleavage agent	References
4-Hydroxymethylphenylacetic acid (PAM) 	Acid	Strong acid	Mitchell *et al.* (1978)
4-Hydroxymethylphenoxyacetic acid (HMPA) 	Acid	TFA	Sheppard and Williams (1982)
4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB) 	Acid	dilute TFA	Florsheimer and Riniker (1991)
4-(2',4'-Dimethoxyphenyl-hydroxymethyl)methylphenoxyacetic acid (Rink acid) 	Acid	dilute TFA	Rink (1987)
5-(4-Hydroxymethyl-3,5-dimethoxyphenoxy) valeric acid (HAL) 	Acid	dilute TFA	Albericio and Barany (1991)

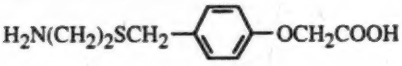
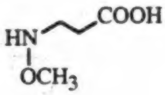
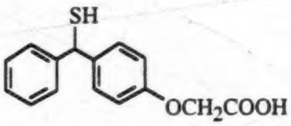
Handles drawn with the point of peptide attachment on far left and resin attachment on far right

Table 3 Selected Resin Handles (continued)

Handle	Resulting C-terminal	Cleavage agent	References
3-Nitro-4-hydroxymethyl benzoic acid (ONb) 	Acid	hν (350 nm)	Rich and Gurwara (1975)
5-(4-Aminomethyl-3,5-di-methoxy-phenoxy)valeric acid (PAL) 	Amide	TFA	Albericio and Barany (1987)
5-(9-Aminoxanthen-2-oxy) valeric acid (XAL) 	Amide	dilute TFA	Sieber (1987)
3-Nitro-4-aminomethyl benzoic acid (Nonb) 	Amide	hν (350 nm)	Hammer *et al.* (1990)
4-(2',4'-Dimethoxyphenylamino-methyl)methylphenoxyacetic acid (Rink amide) 	Amide	dilute TFA	Rink (1987)

Handles drawn with the point of peptide attachment on far left and resin attachment on far right

Table 3 Selected Resin Handles (continued)

Handle	Resulting C-terminal	Cleavage agent	References
4-(2-Aminoethylmercapto)-methylphenoxyacetic acid 	N-mercapto-alkylamides	HF	Engelbretsen *et al.* (1995)
3-(N-methoxyamino)propionic acid 	Aldehydes	$LiAlH_4$	Fehrentz *et al.* (1995)
4-(α-Mercaptobenzyl)phenoxy-acetic acid 	Thioacids	HF	Canne *et al.* (1995)

Handles drawn with the point of peptide attachment on far left or top center and resin attachment on far right

Figure 10 Oxazolone Formation and Racemization

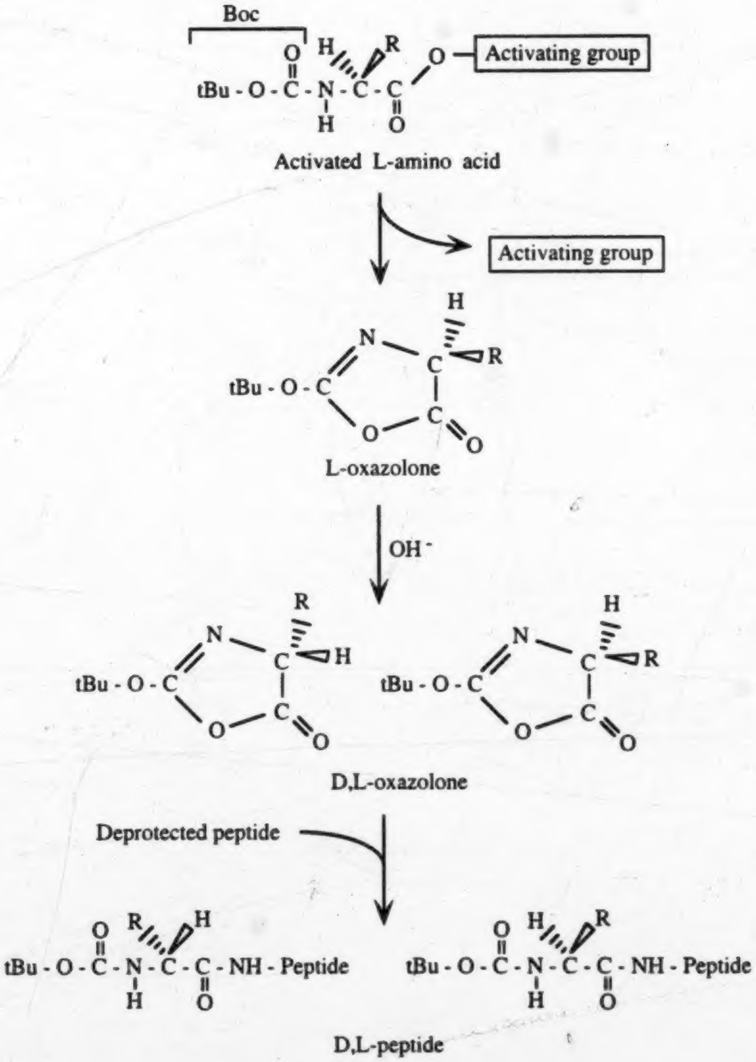
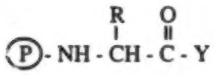

Boc
Activating group
Activated L-amino acid
Activating group
L-oxazolone
OH⁻
D,L-oxazolone
Deprotected peptide
NH - Peptide
NH - Peptide
D,L-peptide

Figure 11 Pre-formed Activated Acid Halides and Active Esters

Acid halides

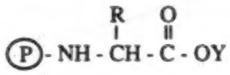


$$\textcircled{P}\text{- NH - }\overset{\overset{\displaystyle R}{|}}{\text{CH}}\text{ - }\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{ - Y}$$

Y = Cl Acid chloride (Fmoc only)

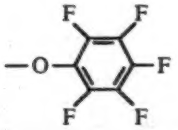
= F Acid fluoride

Active esters

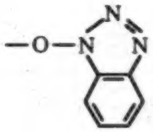


$$\textcircled{P}\text{- NH - }\overset{\overset{\displaystyle R}{|}}{\text{CH}}\text{ - }\overset{\overset{\displaystyle O}{\|}}{\text{C}}\text{ - OY}$$

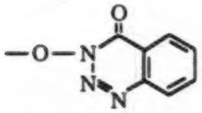
OY =



OPfp



OBt



ODhbt

$\textcircled{P}$ = Urethane N^{α}-protecting group (Boc or Fmoc)

Figure 12 **Carbodiimide Activation and Peptide Bond Formation**

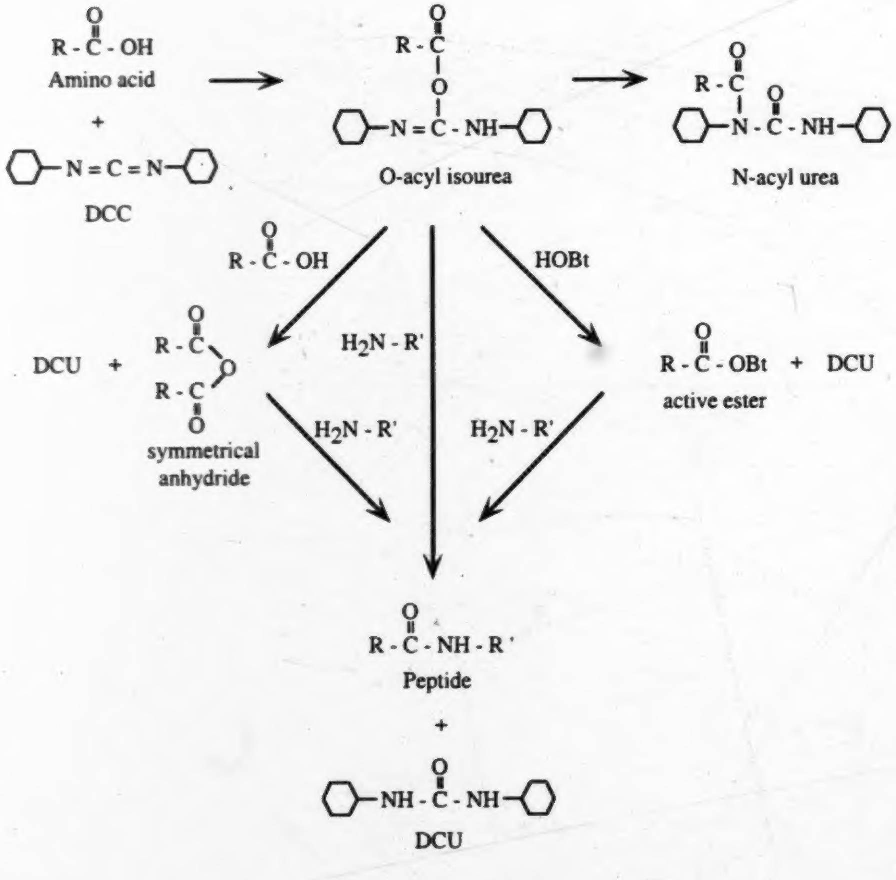

R - C - OH
Amino acid
+
N = C = N
DCC
R - C
O
N = C - NH
O-acyl isourea
R - C
N - C - NH
N-acyl urea
R - C - OH
HOBt
DCU +
R - C
R - C
symmetrical anhydride
H_2N - R'
H_2N - R'
H_2N - R'
R - C - OBt + DCU
active ester
R - C - NH - R '
Peptide
+
NH - C - NH
DCU

Figure 13 Structures of Phosphonium *In situ* Coupling Reagents

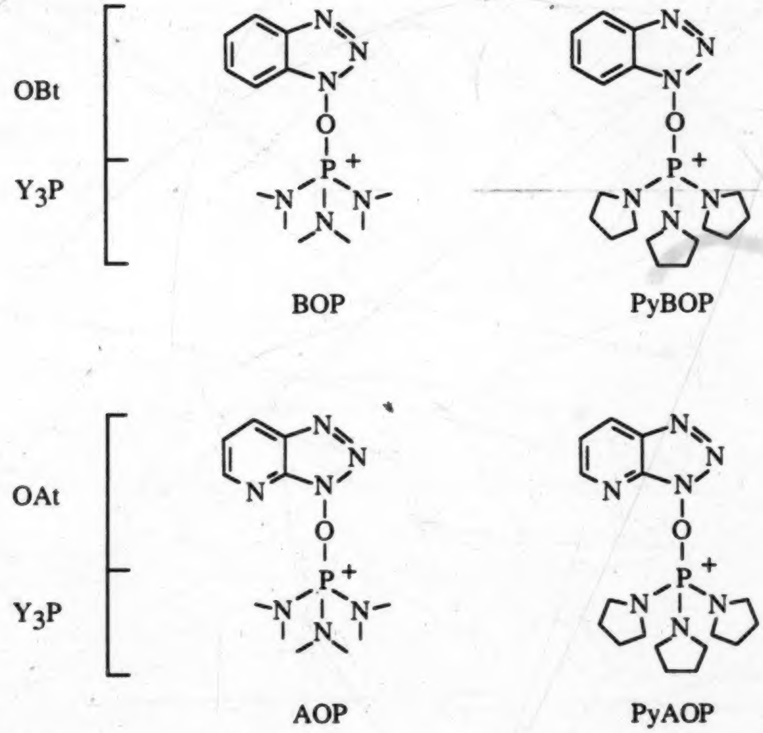

OBt
Y3P
N
N
N
O
P+
N
N
N
BOP
PyBOP
OAt
Y3P
AOP
PyAOP

Figure 14 Formation of OBt and OAt Active Esters From Phosphonium *In situ* Coupling Reagents

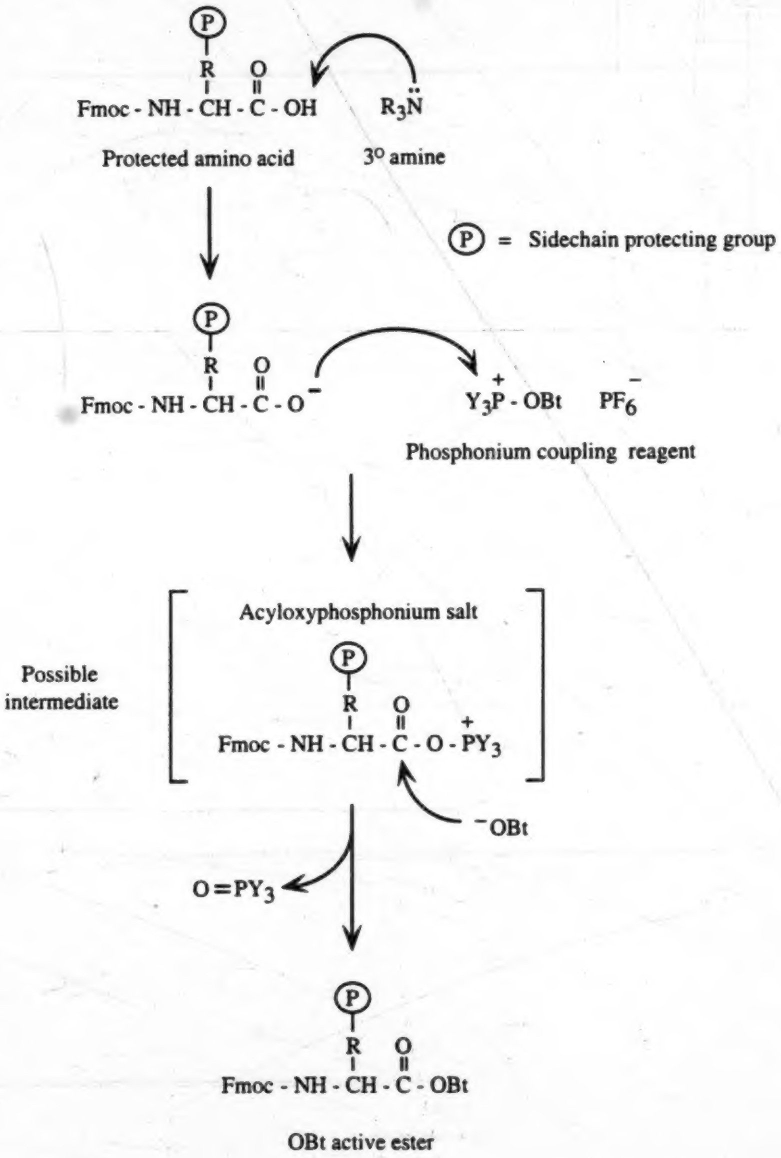
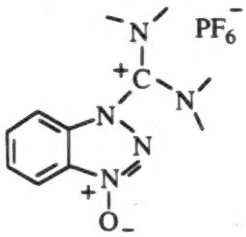
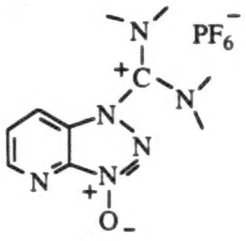

P
R
O
Fmoc - NH - CH - C - OH
R3N
Protected amino acid
3° amine
P = Sidechain protecting group
P
R
O
Fmoc - NH - CH - C - O−
Y3P+ - OBt PF6−
Phosphonium coupling reagent
Possible intermediate
Acyloxyphosphonium salt
P
R
O
Fmoc - NH - CH - C - O - PY3+
−OBt
O=PY3
P
R
O
Fmoc - NH - CH - C - OBt
OBt active ester

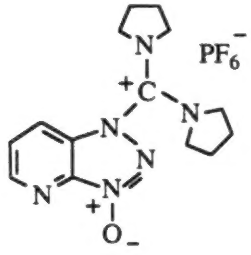
Figure 15 Structures of Aminium *In situ* Coupling Reagents



HBTU



HATU



HAPyU

TBTU and TATU are the equivalent tetrafluoroborate (BF_4^-) salts

Figure 16 Acidolytic Cleavage of Peptide From Resin

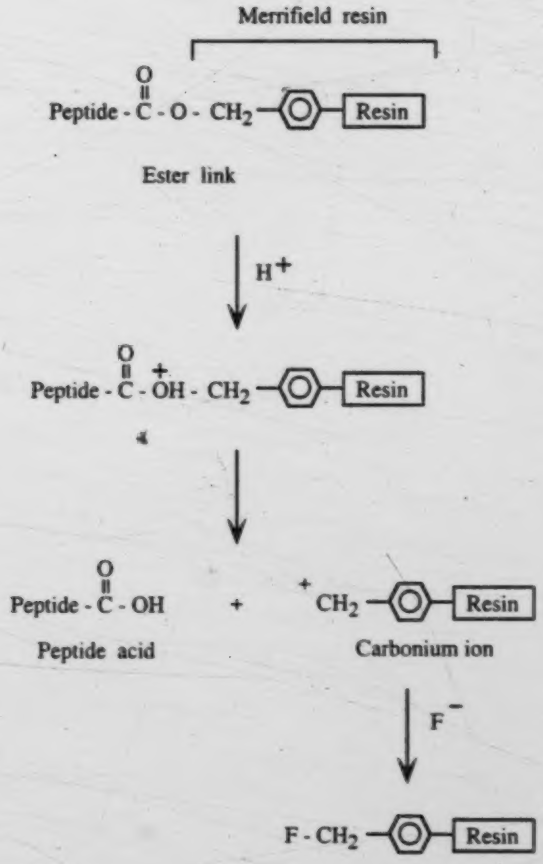
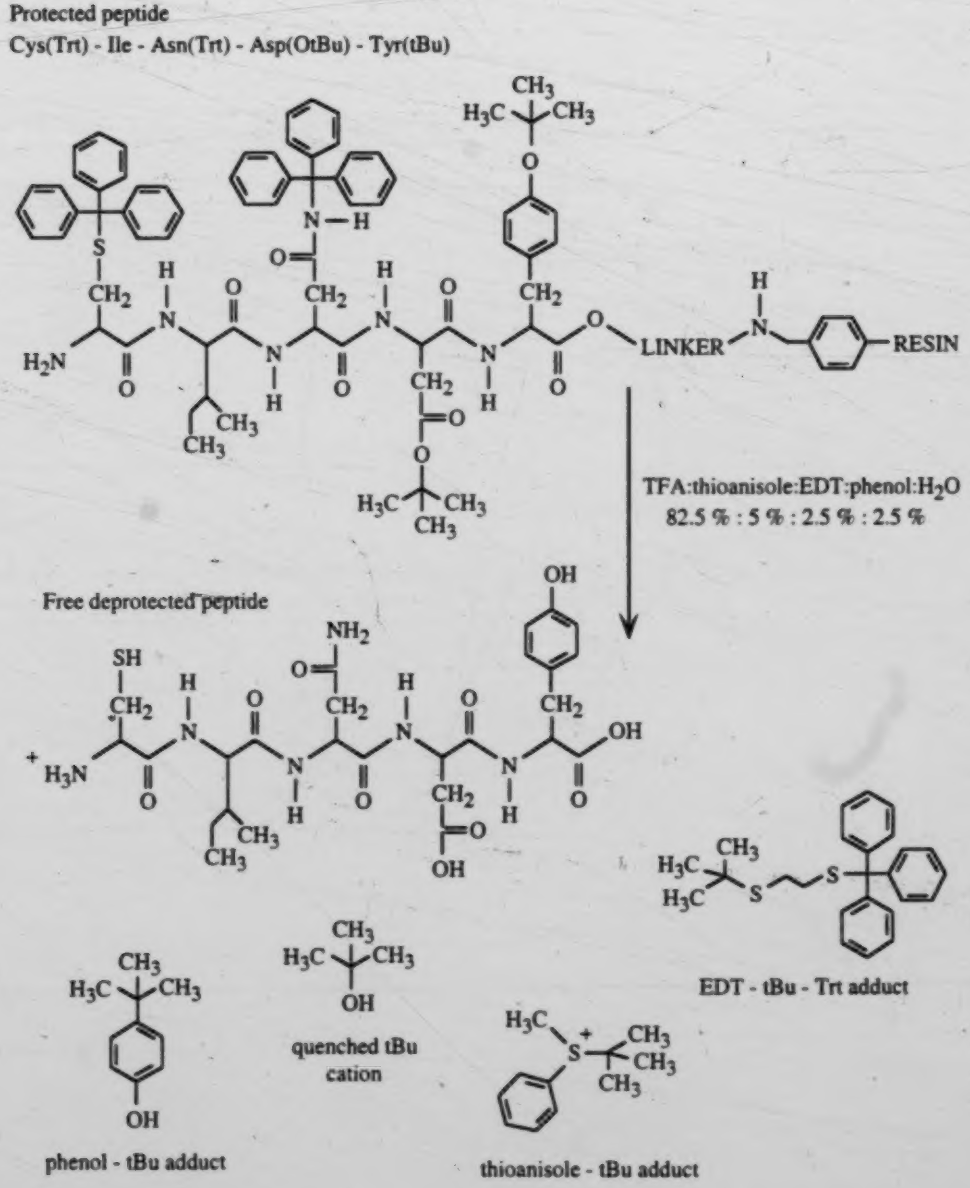

Merrifield resin
Peptide - C - O - CH2
Resin
Ester link
H+
Peptide - C - OH - CH2
Resin
Peptide - C - OH
+
CH2
Resin
Peptide acid
Carbonium ion
F-
F - CH2
Resin

Figure 17 Possible Reactions of TFA and Scavengers With Protected Peptide

Guy and Fields (1997)


Protected peptide
Cys(Trt) - Ile - Asn(Trt) - Asp(OtBu) - Tyr(tBu)
LINKER
RESIN
TFA:thioanisole:EDT:phenol:H2O
82.5 % : 5 % : 2.5 % : 2.5 %
Free deprotected peptide
EDT - tBu - Trt adduct
phenol - tBu adduct
quenched tBu cation
thioanisole - tBu adduct

2.2 Development of Multiple Peptide Synthesis

One of Merrifield's goals in developing SPPS was the automation of peptide synthesis based on the simplicity and speed of the repetitive steps in chain elongation, which can be carried out in a single reaction vessel at ambient temperature. The development of an automatic peptide synthesizer required a suitable reaction vessel to contain the resin and to provide for its mixing (by inversion, vortexing, or nitrogen bubbling) with reagents and wash solvents, a pressurized system for selecting, metering and transferring the correct solvent or reagent from the appropriate reservoir to the reaction vessel with a minimum of cross contamination and removing reagents or solvent from the vessel after an appropriate time interval, and a programmer for sequencing and timing all operations. Numerous models for both batchwise and continuous flow operation at various scales of operation (generally 0.1-1.0 mmol) have been offered since the introduction of the first synthesizer in 1966, some with continuous monitoring capability. Both Boc and Fmoc strategies have been accommodated (although only the latter is appropriate for continuous flow operation), and coupling methods include the use of pre-formed activated amino acid derivatives, pre-activation, or *in situ* coupling reagents. These synthesizers allow virtually unattended peptide production, interrupted only by the requirements for initial set-up and manual cleavage at the end of each synthesis. Yet despite the reduction in synthesis time and the increased efficiency, these conventional instruments are still limited to performing individual peptide syntheses in a serial manner (or at most, a few simultaneous syntheses using several continuous flow reaction columns).

Initial attempts at developing multiple peptide synthesis (MPS) were driven by the escalating demand for synthetic peptides for immunological screening - the inability of serial synthesis to meet the demand for large numbers of peptides in a reasonable time frame necessitated the development of alternate methods based on parallel (i.e. simultaneous) synthesis. Very small amounts of peptide (in the nmol range) are often sufficient for screening purposes, reducing initial demands on multiple peptide synthesis methodology and instrumentation. This concept was refined in the **Pin method** (Geysen *et al.*, 1984), which recognized that solution-phase immunoassays could be replaced by solid-phase testing in which the same solid support could be used for both peptide synthesis and immunological screening. This allowed synthesis to be limited to the production of very small amounts of individual peptides retained on the support for subsequent screening, removing only the N^{α} and sidechain protecting groups required during synthesis. In the Pin method, up to 96 peptides can be prepared simultaneously on a novel solid support consisting of functionalized polyethylene or polypropylene rods or "pins" radiation-grafted with methacrylic acid/dimethylacrylamide and assembled in a polyethylene holder in the 8x12 array format and spacing of a standard 96 well microtitre plate used in immunoassays (Bray *et al.*, 1990). Washing and deprotection steps are carried out collectively in baths, whereas amino acid coupling is performed individually in the wells of solvent-resistant microtitre plates by immersing the pins. Since peptides are support-bound both during and after synthesis, the binding capability of antibodies can be investigated by placing the entire block on an ELISA (enzyme-linked immunosorbent assay) plate and incubating the pin-peptides in antibody solutions distributed in the plate wells. After testing, it is usually possible to regenerate the peptides for repeated screening by disrupting the peptide-antibody complex. This method has been developed extensively by Chiron Mimotopes (Clayton, Victoria, Australia) and is marketed as the "Multipin System". The major limitation to the support-bound peptide approach is that many molecular interactions of interest involve membrane-bound (i.e. solid-phase) receptors interacting with solution-phase peptides or proteins in the surrounding environment.

Recent modifications include increased peptide capacity and the introduction of cleavable handles on the pins to facilitate the generation of free peptides in larger quantities for solution-based assays. However, this MPS system remains directed towards rapid solid-phase immunological testing, and the low (i.e. μmol) peptide yield may well be insufficient for solution-phase research applications.

Spot synthesis was devised as an inexpensive alternative to the original Pin method for generating surface-bound arrays of peptides targeted for immunological testing. In this approach, peptides were synthesized as 8x12 arrays of discrete spots on functionalized support sheets replacing the polypropylene rods of the Pin method, and these sheets were directly employed in screening. The simplest method utilized cellulose sheets (Frank *et al.*, 1991), whereas a more elaborate procedure made use of polypropylene membranes for greater ease of handling and stability (Wang and Laursen, 1992). In the latter case, the membrane was first sandwiched between two aluminum templates with holes bored in the standard array of a 96-well microtitre plate, pierced in the center of each hole, and mounted on a polypropylene vacuum chamber sealed by a rubber gasket. In both methods, coupling was accomplished by applying small aliquots of solution directly to the spots. Cellulose sheets were washed and deprotected by immersion in baths, but in the case of the polypropylene membranes the deprotection and wash solutions were pipetted onto the membranes and removed by evacuation of the vacuum chamber. In both methods, the focus was directed to support-bound immunological testing, although small quantities of free peptide could be obtained from the support by punching out the spots and cleaving them individually.

Light-directed, spatially addressable synthesis (Fodor *et al.*, 1991) is the most technologically advanced addition to the collection of syntheses generating permanent support-bound peptide arrays (or indeed, to the entire range of multiple peptide syntheses). This method employs light to direct solid-phase synthesis on a glass microscope slide. Photolabile N^{α}-protection and a computer-controlled masking system (used during irradiation to accomplish selective deprotection) determines which regions of the solid support are activated for coupling. The pattern of masks and the sequence of amino acid coupling defines the resulting surface-bound peptides and their locations. Immunological screening by fluorescence immunoassay can detect and identify peptides by the location of the fluorescing region on the slide. The method is targeted towards the creation of large peptide libraries: the number of peptides which can be synthesized is limited only by the number of synthesis sites which can be addressed with appropriate resolution (i.e. 10,000 per cm^2), conferring a high degree of miniaturization. Binary masking (one of many possible combinatorial synthesis strategies which have been developed) yields 2^n peptides in n steps. Since the position of each peptide in the library is known (i.e. spatially addressed), fluorescence immunoassays can provide rapid identification from the location of fluorescing regions on the slide. Potential problems with this method include incomplete photolytic deprotection during synthesis and the possibility of multivalent antibody-peptide interactions during screening (due to high peptide density) so that fluorescence may give only a qualitative indication of binding affinity. Designed to create vast peptide diversity on a miniature scale rather than generate significant quantities of individual peptides, this new and expensive technology is not yet appropriate for routine peptide synthesis, and may find its true role in biosensor research.

Methods for generating support-bound peptides such as those described above were devised to provide large peptide arrays suitable for rapid immunological testing, and the success of the Multipin System verifies this design approach. However, even with modifications such as the incorporation of cleavable handles on the support and procedures to allow free peptide recovery, these methods cannot generate sizeable yields of individual peptides. Alternate approaches have been developed to provide free peptides on a scale suitable for solution-based studies. The **Tea Bag method** (Houghten, 1985) is one of the oldest strategies for multiple peptide synthesis and remains in current use, especially for the creation of peptide libraries. The method is capable of generating 50 mg quantities of up to 150 peptides. Small amounts of polymeric support are sealed in polypropylene mesh bags, and these resin packets are brought through collective deprotection and washing steps and are separated for coupling and cleavage. Prior to each coupling step the "tea bags" must be sorted according to amino acid residue addition, and then reacted in parallel in separate reaction vessels. This method not only provides high yields but also allows flexibility in the choice of coupling schemes and solvents, since separate reaction vessels are used. However, the labour component is significant due to the number of mechanical operations with the tea bags. The Tea Bag method has been semi-automated for washing (Beck-Sickinger *et al.*, 1991) and commercialized by Biotech Instruments. A variant on the Tea Bag method employed sheets of long-chain polystyrene grafted on to polyethylene film in place of resin packets for the solid support (Berg *et al.*, 1989). The **Film method** eliminated the labour involved in preparing the solid support (other than cutting up film), but individual pieces of film still required sorting and parallel coupling in separate reaction vessels. Otherwise, the two methods were similar and peptide yields on PS-PE film were comparable to those of the Tea Bag method.

The **Cellulose Disk method** (Frank and Döring, 1988) was also based on a redistributed solid support, but was operated as a continuous flow version. Peptides were synthesized on functionalized cellulose paper disks of 3 mm thickness stacked in a reactor column to allow continuous flow operation. Peptides could be retained on the cellulose support or cleaved for use as free peptides. Redistribution of the solid support during synthesis followed a "segmental support approach" in which those disks which required the same amino acid coupling were stacked together in a column to undergo the reaction together. Washing and deprotection steps were also performed collectively in the column under continuous flow conditions. Up to 100 disks could be accommodated in a single column, and several columns could be operated in parallel. Once again it was not possible to automate redistribution of the solid support, and a great deal of organization was required to sort disks and stack columns according to the amino acid to be coupled. Collective coupling in a column also imposed limited flexibility in the choice of coupling schemes, and individual peptide yields of only 8-10 mg restricted potential applications.

A different version of the segmental support approach stacked individual reactor columns in series and operated the system in continuous flow mode under low pressure conditions (Krchnák *et al.*, 1989). Each column consisted of a 5 mL polypropylene syringe, and up to 10 columns could be joined in series. This system was designed for the synthesis of analogous peptides, where most couplings could be performed collectively in continuous-flow mode and very few required disconnection of the individual reactors for batch-mode coupling of individual amino acids. For synthesis of completely different peptides, all reactors had to be disconnected for manual coupling in batch-mode. For this reason, automation was not possible.

Fully automated multiple peptide synthesis was finally achieved with the use of robotic sample processors, i.e. pipetting robots modified and programmed for the requirements of peptide synthesis. The version developed by Schnorrenberg and Gerhardt (1989) employed a PC-controlled pipetting robot equipped with two independent arms, one for serial reagent addition and the other for serial washing. Amino acids were dispensed by needle from a reagent rack of glass vials into another rack of test tubes or Eppendorf tubes serving as reaction vessels. The washing arm was equipped with two needles, one for solvent addition and the other (fitted with a stainless steel sieve) for solution removal by suction. This allowed for the simultaneous synthesis of a large number of peptides (up to 96) at a scale useful for solution-based studies. Another version developed by Gausepohl *et al.* (1990) utilized a single arm pipetting robot for reagent addition with the advancement of simultaneous rather than serial distribution, employing six needles on the arm for row-by-row reagent transfer to a 6x8 array of reaction vessels. In place of a second washing arm, each reaction vessel (a polypropylene tube) was equipped with a bottom sintered glass frit to allow solution removal by suction applied through a vacuum pump. Yet another version developed by Nokihara *et al.* (1992) supplied the amino acids, solvents and reaction vessels on eight tracks. Each track was used for the synthesis of one peptide and contained the complete set of reagents for a synthesis; the single arm of the pipetting robot was equipped with eight needles for simultaneous reagent delivery. Unfortunately, this version was limited in that it did not allow any expansion in the number of peptides to be synthesized.

None of the previously described MPS systems met all the project requirements for a simple, reliable and inexpensive multiple peptide synthesizer capable of generating large numbers of peptides in reasonable quantities for solution-based studies (i.e. at least 20 mg per peptide), and the problem of multiple cleavage was only beginning to be addressed. Support-bound systems removed the need to track individual peptides during synthesis, but could not operate at a suitable production scale. The Tea Bag, cellulose disk, and stacked syringe methods were designed to operate at a greater scale of synthesis, but the considerable labour required for sorting operations prior to batch coupling precluded automation. Continuing development of multiple peptide synthesis and appropriate instrumentation parallel with this project has focussed on automation using robotic pipettors (even though this retains a serial component to peptide synthesis), and recently more elaborate programmable synthesizers with completely enclosed systems in the fashion of conventional (i.e. single peptide) synthesizers have become available commercially. However, this approach greatly increases the cost of a multiple peptide synthesizer and will restrict accessibility to large-scale users such as pharmaceutical companies or service facilities.

Chapter 3 DESIGN OF A NOVEL MULTIPLE PEPTIDE SYNTHESIZER

3.1 Design Process

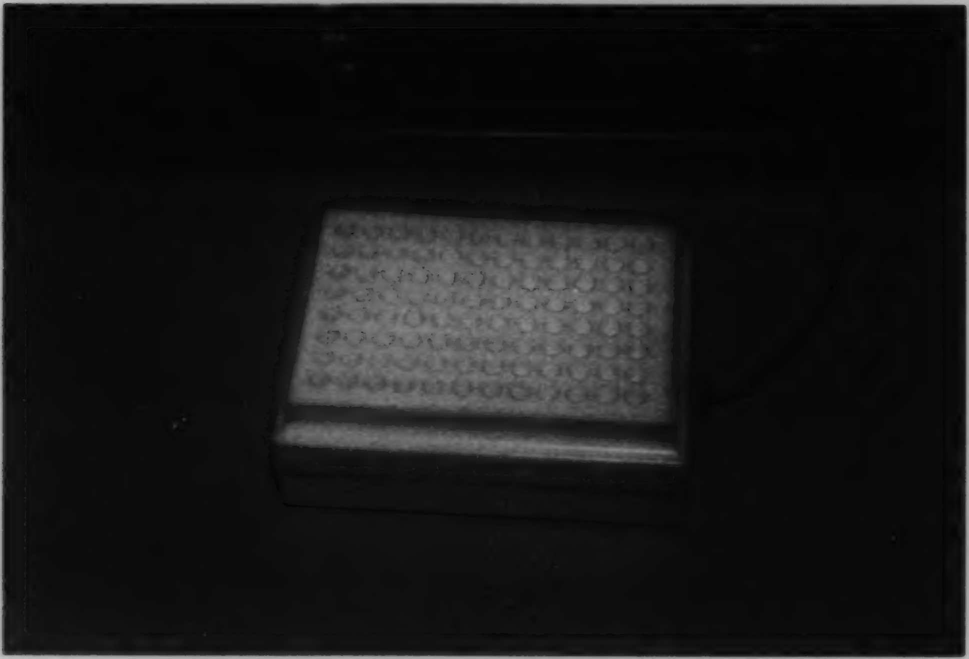
An alternate approach to multiple peptide synthesis was devised by Holm and Meldal (1989), combining an increased production scale (made possible by the use of dedicated reaction vessels for peptide synthesis) with the convenience of the array format utilized by support-bound methods. This approach eliminated the sorting manipulations (and peptide tracking) required in the Tea Bag and other similar methods prior to common coupling steps, by distributing the coupling solution amongst reactors in the array rather than redistributing solid-support between coupling baths. The introduction of liquid distribution manifolds for common washing and deprotection steps and a valveless reactor evacuation system provided a simple but effective means of conferring automation to a degree achieved only later by robotic pipettors at far greater expense. The key concepts illustrated by the resulting laboratory instrument served as the starting point in this synthesizer design project.

The Holm and Meldal synthesis unit was based on the 96-well microtitre plate used in immunoassays, and consisted of a small teflon block of approximately the same dimensions (130 mm length x 95 mm width) and format as the plate **(Photograph 1)**. The upper section of the block contained 96 "wells" in the standard 8x12 array and 9 mm spacing of the microtitre plate. These cylindrical "wells" (6 mm diameter, 25 mm depth) were actually small column reactors with a volume of approximately 700 μL, draining through narrow (1 mm ID) stainless steel tubes extending from the bottom of each reactor. A bottom teflon frit (2 mm thick, 70 μ nominal pore size) was used to retain solid support for peptide synthesis within each reactor, reducing the working volume to approximately 650 µL. Coupling reagents were added manually to reactors in a manner modelled after immunoassay reagent addition: an 8-channel multi-pipettor was used for column-by-column transfer from reagent tray wells in the standard 8x12 arrangement and spacing. Reactor drainage tubes protruded 5 mm below the upper section into a base which directed waste to an outlet port through a series of milled grooves or channels **(Photograph 2)**. The two sections of the synthesis unit were sealed by a silicone rubber gasket and held together by screws inserted from the bottom of the base **(Photograph 3)**. A unique feature of this design was the ability of the base to function as a vacuum chamber (assisting reactor drainage) as well as a reactor "shut-off" system (base pressurization eliminating the need for individual reactor valves); a version of this vacuum chamber was seen later in combination with spot synthesis on polypropylene membranes (Wang and Laursen, 1992). Another desirable feature was the semi-automation of common washing and deprotection steps through the introduction of two dedicated overhead liquid distribution manifolds for simultaneous delivery of solvent or deprotection reagent to reactors. Each manifold was constructed from two teflon blocks, with a silicone gasket spacer ensuring the formation of a liquid layer between the blocks; simultaneous delivery to the reactors in the synthesis unit was performed through a series of stainless steel tubes (0.5 mm ID) extending into the liquid reservoir between the blocks and protruding from the underside of the manifold in the standard 8x12 arrangement. As in all MPS systems described previously, no provision was made for multiple cleavage; this labour-intensive procedure was carried out in small glass culture tubes (6 x 50 mm) inserted tightly into the reagent tray **(Photograph 4).** Transfer of the thoroughly dried support-bound peptides from the reactors to the cleavage vessels was accomplished by positioning the reagent tray upside down over the synthesis unit (using the raised edges of the tray to grasp the synthesis unit chamferred edges), upending the combination, and tapping the synthesis unit to ensure complete transfer of the support before removal of the unit **(Photograph 5)**. Resins were cleaved individually, and the resulting peptide solutions were removed by pipet for further processing and peptide recovery.

The adoption of key design concepts in this instrument (i.e. the array format of the reactors, the dual purpose base, and the use of a liquid distribution manifold), a significant increase in production scale (with attendent technical problems to be solved), and the design of a multiple cleavage system to complement the multiple peptide synthesizer were critical elements in the development of a commercial multiple peptide synthesizer prototype. Column reactors were retained in the design process, but the microtitre plate format, while convenient for manipulation, was insuitable for production scaleup. Two reactor array formats differing in reactor number were selected to accommodate a range of synthesis projects. Column width and length were approximately doubled to provide a 7-fold increase in reactor volume, which represented a compromise between increasing production scale and growing demands on the base pressurization system to hold solutions within the reactors. A multiple peptide cleavage system was designed on the basis of dual-purpose column reactors suitable for both synthesis and cleavage, using Fmoc chemistry and TFA as the cleavage reagent. A greatly expanded base with a large interior chamber was constructed to accommodate a removeable, chemically resistant test tube rack during cleavage (for the collection of cleaved peptide solutions), while maintaining its role as a vacuum/pressurization chamber. The increased demands on the base pressurization system caused by the greater reactor volume, the large interior chamber of the collection base, and the tendency of TFA to "creep" posed the greatest problem; this was eventually overcome by the combination of smaller reactor drainage tubes (0.5 mm ID), an improved seal between reactor block and collection base (formed by a more durable and chemically resistant o-ring in place of a sealing gasket) and the use of adjustable tension clamps (in place of cumbersome screws) for assembly of the reactor block and base into a reaction module. Potential corrosion problems caused by TFA led to the choice of glass rather than stainless steel reactor drainage tubes, selecting thick-walled capillary tubing to minimize breakage and address handling concerns; reactor drainage tubes were recessed to provide further protection and improve safety during handling (adding to reactor block dimensions). Alternate block materials with suitable chemical resistance were investigated due to the prohibitive expense of teflon. A multi-purpose distribution manifold was developed from the original Holm and Meldal design for dispensing washing, deprotection, or (where desired) cleavage solutions simultaneously to reactors in the reaction module. The various elements of the multiple peptide synthesizer design were incorporated into an open cabinet equipped with a control panel for valves and distribution systems, a "working area" for manual operations with the reaction module, and a dispensing station with easy sideways access for the reaction module; the open cabinet format was chosen to ensure easy access, minimize damage caused by chemical spills, and reduce material costs.

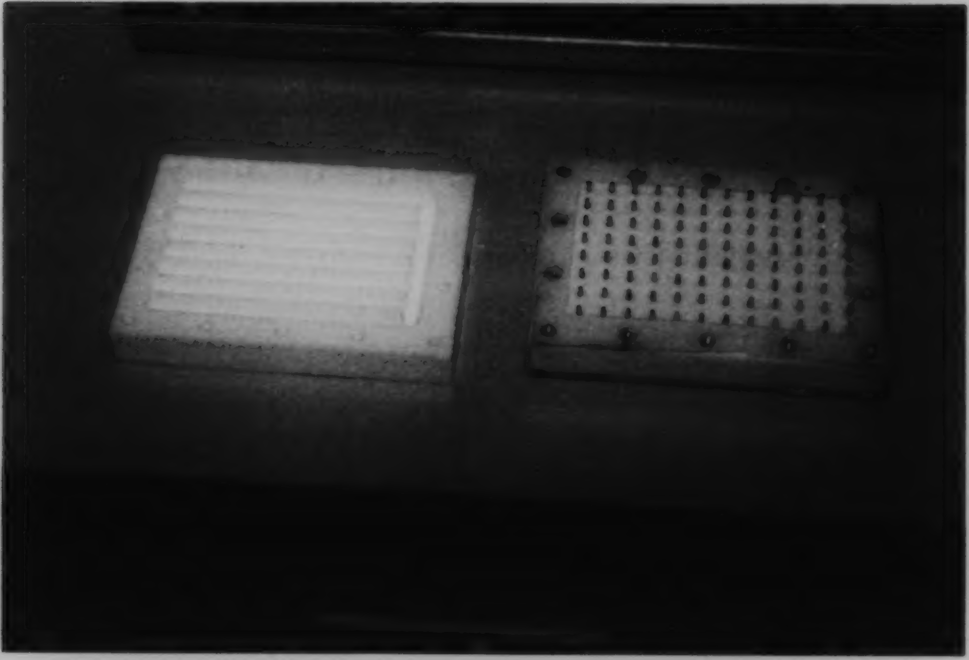
The design process generated a simple and affordable semi-automated multiple peptide synthesizer with multiple peptide cleavage capability and the potential for further low-cost automation by placing the dispensing station under microprocessor control.

Photograph 1 **Holm and Meldal synthesis unit, showing reactors in 8x12 array of a microtitre plate**

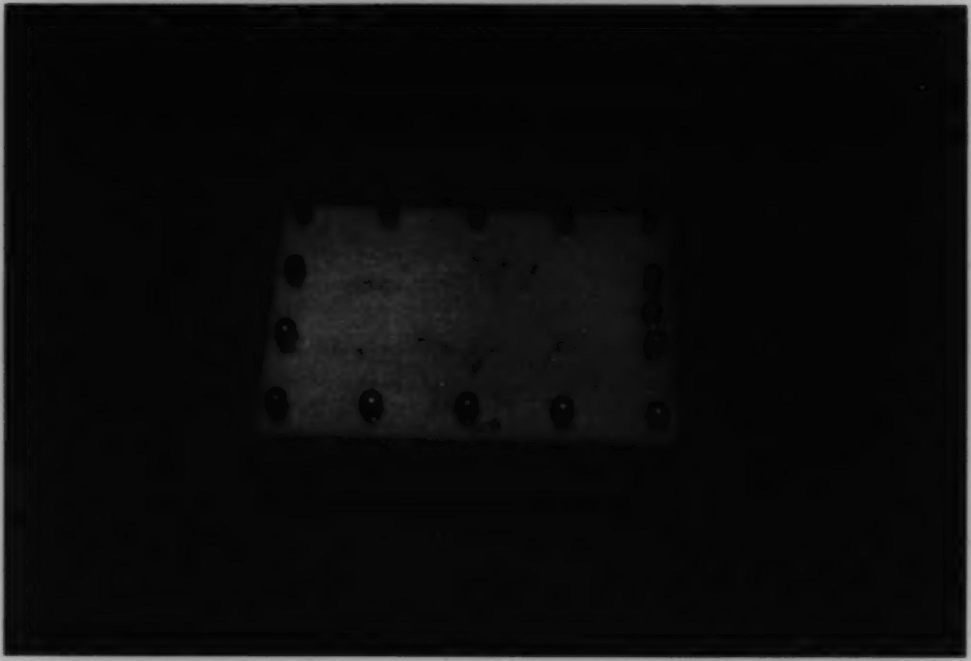


Photograph 2 **Interior of synthesis unit**

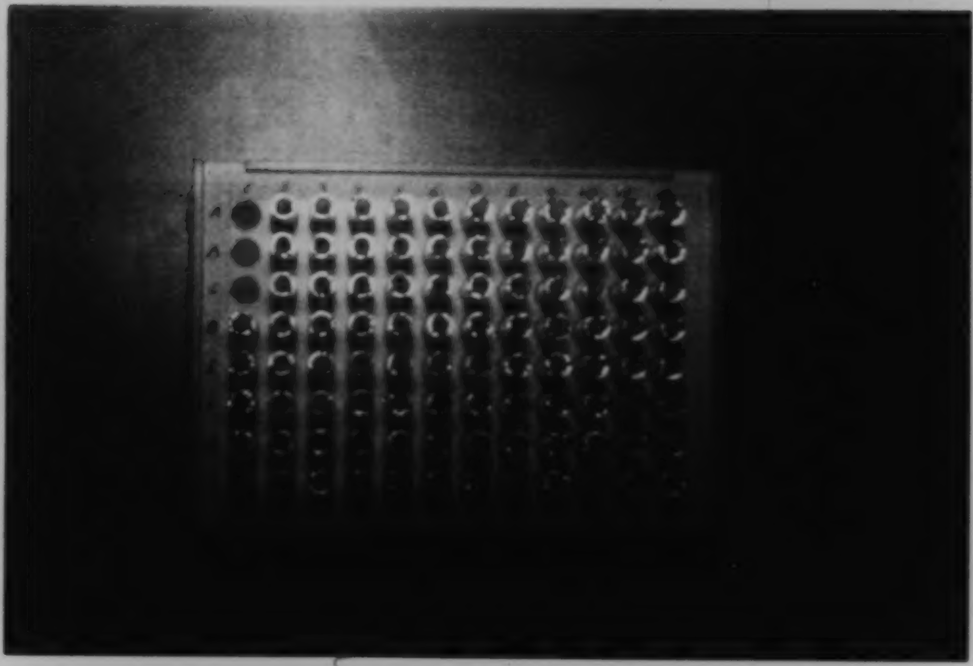
Right: Underside of well section with protruding drainage tubes
Left: Grooved collection base



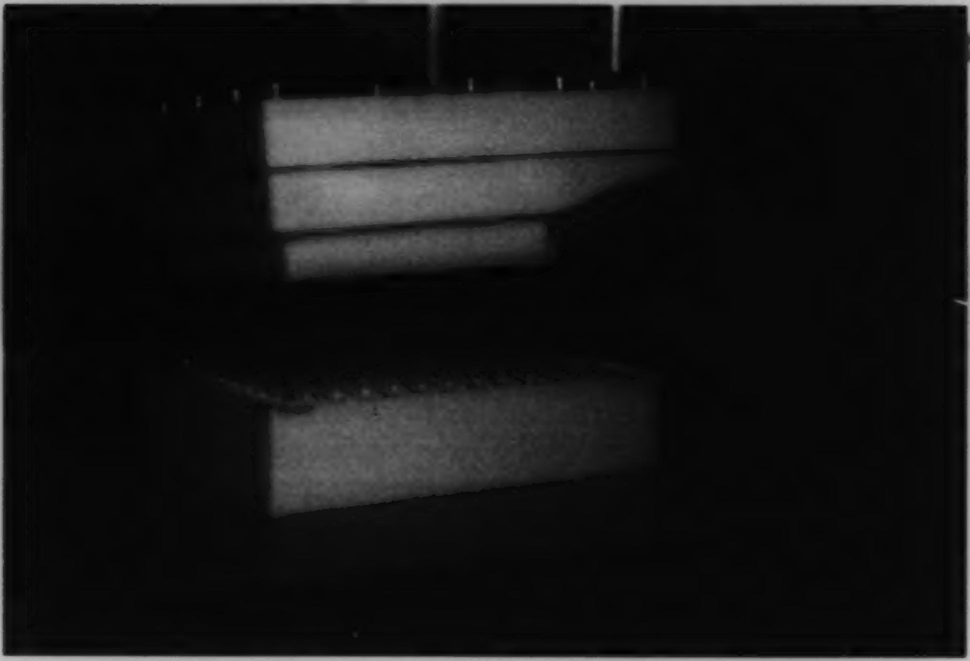
Photograph 3 Bottom of synthesis unit, showing screws holding sections together



Photograph 4 Reagent tray with glass vials in place for cleavage



Photograph 5 Transfer of resin from synthesis unit to cleavage vials



3.2 Technical Description of Prototype

The multiple peptide synthesizer prototype (alpha-test version) resulting from the design process is presented in **Photograph 6,** with an accompanying schematic representation in **Figure 18**. The semi-automated synthesizer is designed to operate in a standard fumehood dedicated to peptide synthesis, and its service requirements include a water tap equipped for aspiration and a nitrogen gas cylinder for pressurization purposes. Two models are available: a 20-reactor version for general use (conveniently designed for analogue synthesis using the 20 naturally occurring amino acids), and a 100-reactor version for routine synthesis of large numbers of peptides or for the creation of peptide libraries; the overall width, depth, and height of the two models are 450 mm x 230 mm x 390 mm (17.5" x 9" x 15.5") and 635 mm x 330 mm x 465 mm (25" x 13" x 18"), respectively. Each model consists of an open cabinet with a conveniently located back control panel, a working area for the reaction module on the left, and a dispensing station on the right, with wash solvent and deprotection reagent reservoirs located to the right of the cabinet and an aspirator trap for waste collection located to the left.

The chemically resistent 316 stainless steel cabinet is equipped with a bubble level on the dispensing station and adjustable feet to ensure the dispenser module mounted in the station remains level. Manually-operated on/off miniature (1.5 mm bore) teflon valves encased in two distribution manifolds (Omnifit, New York, U.S.A.) are mounted on the back panel, one controlling nitrogen gas flow,vacuum and access to the reaction module, and the other controlling solvent and deprotection reagent flow and access to the dispensing station. The nitrogen gas flowrate can be precisely regulated to a maximum 1.0 L/min using a panel-mounted gas rotameter equipped with a fine-control valve and positioned in advance of the reaction module manifold. A single on/off valve controls vacuum access to the dispensing station for evacuation and cleaning purposes. Pressurization and evacuation of the reaction module are performed through a common teflon tubing line (0.5 mm ID, 1.5 mm OD). Solvent and deprotection reagents are supplied to the dispenser through individual teflon tubing lines (0.5 mm ID, 1.5 mm OD) using manually-operated bottletop dispensers mounted on the 4L amber glass bottle reservoirs. All line connections are made with flangeless polypropylene nut and PTFE ferrule connectors (Omnifit, New York, U.S.A.) to allow repetitive connection/disconnection and rapid replacement.

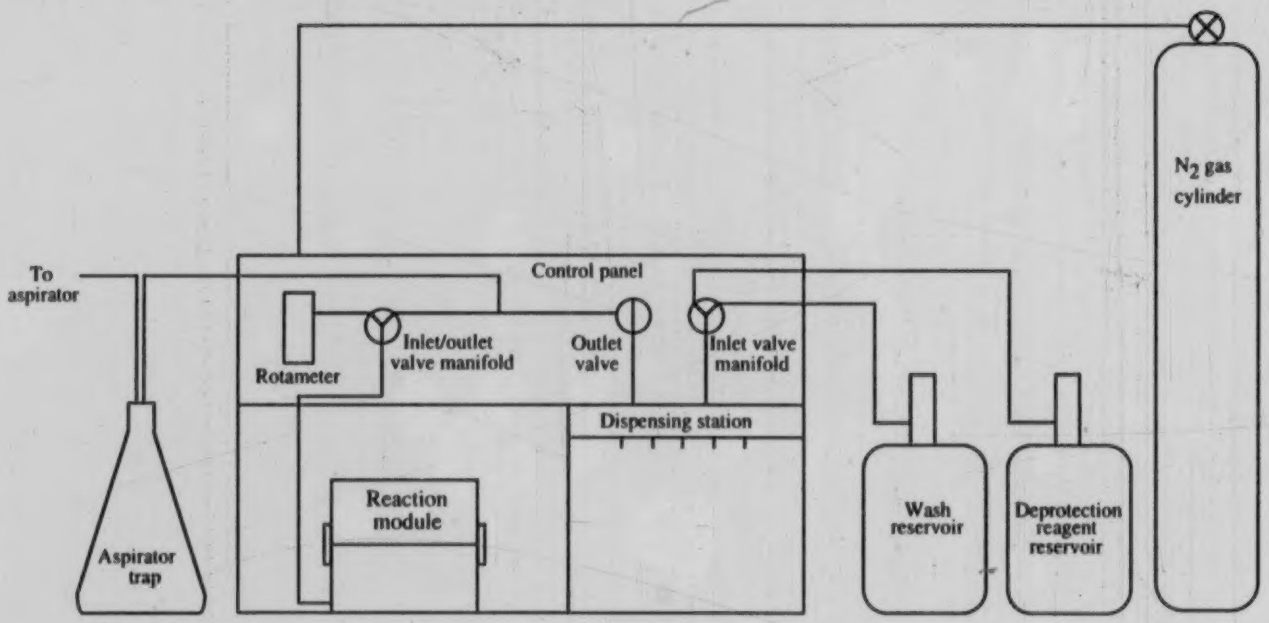
The dual-purpose reaction module is designed to perform peptide synthesis and cleavage in a single unit, and detailed technical drawings are supplied in **Appendix A**. The 20-reactor module has a width, depth and height of 140 mm x 122 mm x 180 mm (5.5" x 4.75" x 7"), while the equivalent dimensions of the 100-reactor module are 230 mm x 230 mm x 180 mm (9" x 9" x 7"). Each inert polypropylene module is assembled from an upper reactor block and a lower hollow collection base. The reactor block contains multiple columns in a 4x5 array or a 10x10 array, depending on the model (as seen in **Photograph 7**); reactors are labeled by column (letter designation) and row (number designation) to aid peptide identification and reagent addition. Each column reactor has a diameter of 13 mm and holds an approximate volume of 5 mL, and the spacing between individual reactors in each array is 18 mm. Column reactors loaded with solid support (the amount depending upon the synthesis scale) are operated in batch-mode. An outlet at the bottom of each reactor opens into a glass drainage tube, and a disposable polyethylene frit (2 mm thick, 20 μ nominal pore size) placed at the bottom of the reactor column using an insertion rod retains the solid support within the reactor. The recessed drainage tubes are constructed from thick-walled 0.5 mm ID glass capillary tubing, inserted by press-fit, and are designed to prevent contamination of neighboring reactors by reagents with a tendency to "creep". The drainage tubes can be seen in **Photograph 8** of the 100-reactor block underside, which also shows the o-ring of inert and chemically resistent C-flex tubing (1/8" ID, 1/4" OD) sealing the two sections of the reaction module. The hollow base with an outlet port on the left side **(Photograph 9)** serves as a pressurization or vacuum chamber during synthesis, maintaining solutions within the reactors by compensating nitrogen pressure or evacuating liquid out of the bottom of the reactors under reduced pressure; if desired, overpressurization of the base can be used to generate nitrogen bubbles for reactor mixing. A collection tube rack, labeled by row and column and equipped with 13x100 mm disposable polypropylene test tubes (8 mL nominal volume), is inserted into the base for the cleavage procedure, and the asymmetric fit ensures correct alignment of the rack with the reactor array **(Photograph 10)**; the test tubes project above the base into the recessed bottom of the reactor block, partially sheathing the reactor drainage tubes to prevent cross-contamination by spraying during collection of the cleaved peptide solutions. The two sections of the reaction module, correctly aligned by guide pins in the base to prevent mismatch of reactors and collection tubes during cleavage, are clamped together using external 2" stainless steel adjustable tension clamps (Spae-Naur, Kitchener, Ont.): two clamps on the 20-reactor module and four clamps on the larger 100-reactor module.

Reagent addition to the reaction module is performed manually, transferring by pipet from the corresponding tube array in a reagent rack; conical polypropylene centrifugation tubes (15 mL or 50 mL, depending on the scale of synthesis) are recommended as reagent containers which permit rapid vortex mixing. In contrast, the common steps of washing and deprotection are semi-automated, performed simultaneously for all reactors in the module through a dispensing station connected to wash solvent and deprotection reagent reservoirs equipped with manual bottletop dispensers **(Photograph 11)**. Precise alignment of the reaction module and the overhead dispenser is achieved by sliding the module into the dispensing station on a track equipped with a polypropylene block positioning stop **(Photograph 12)**; the minimal clearance between dispenser and reaction module and the moderate rate of flow from the dispenser into each reactor eliminates potential cross-contamination due to spraying. Two models of the dispenser module are provided, corresponding with the two formats of the reaction module, and detailed technical drawings of each are supplied in **Appendix A**. The inert polypropylene dispenser is assembled from an upper section containing a row-and-column array of hemispherical concave depressions on its lower surface and a solid base pierced in the same array format by stainless steel drainage tubes (0.5 mm ID, 0.8 mm OD) extending slightly above the base into the corresponding concave depressions **(Photograph 13)**. A thin solvent chamber is formed between the two sections by a spacing seal consisting of an o-ring composed of inert C-flex tubing (1/8" ID, 1/4" OD), and the two sections are held together by external 1" stainless steel adjustable tension clamps; this arrangement ensures even distribution of liquid through the dispenser to each reactor and prevents bubble locks forming in the drainage tubes, since the tops of the tubes extending into the depressions of the upper section permit trapped air to escape before liquid flow begins. The drainage tubes direct solvent or deprotection reagent into the center of each reactor column in the reaction module stationed below (see **Photograph 14** of the underside of the dispenser module, displaying the recessed tubes in the base). The dispensing station can also be used for simultaneous transfer of cleavage reagent to the reaction module from a third reservoir, or cleavage reagent may be transferred manually by serial pipetting (when more than one type of reagent is desired).

Depending upon the scale of synthesis in each reactor, precipitation of crude peptide from cleavage solutions drained from the reactors may be carried out directly in the 13x100 mm collection tubes or after manual transfer of the cleaved peptide solutions from these collection tubes to 50 mL polypropylene conical centrifugation tubes. While centrifugation can be used to aid collection of the precipitated peptides, it is not essential (especially as the requirement for a volatile solvent-compatible centrifuge may pose a problem). Supernatant removal after precipitation and serial washing of the precipitates is performed by aspiration, and an N-Evap evaporator (Organomation Assoc., U.S.A.) connected to the nitrogen gas cylinder is used to dry the crude peptides in batches **(Photograph 15)**. Tubes containing the wet peptide precipitates are spring-loaded into a holding rack positioned within a 30° C waterbath to compensate for heat loss during evaporation. The evaporator is equipped with 12 hollow needles to direct gas flow into the tubes for forced convection assistance to evaporation; gas flowrate to individual tubes can be adjusted as required during evaporation using separate valves on the gas lines leading to the needles **(Figure 19)**. Lyophilization of the crude peptides can be performed directly in the tubes, which are loaded into a circular holder designed to fit within a standard glass dessicator for attachment to a vacuum manifold (or alternately, for use in a large-scale tray lyophilizer).

Photograph 6 Multiple peptide synthesizer prototype (alpha-test version)

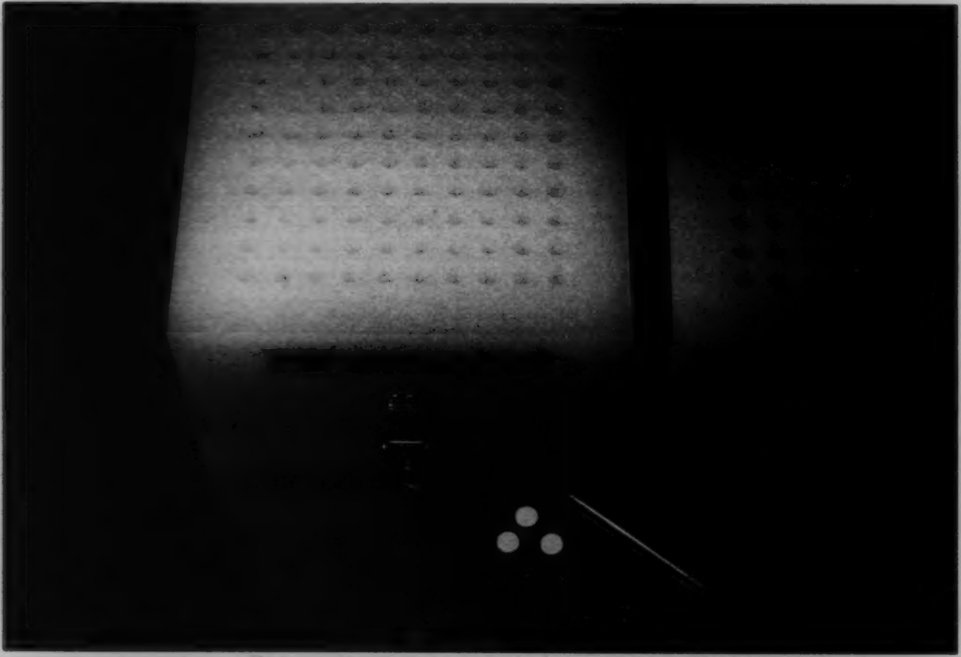
Figure 18 Schematic Representation of Multiple Peptide Synthesizer Prototype


To aspirator
Aspirator trap
Control panel
Rotameter
Inlet/outlet valve manifold
Outlet valve
Inlet valve manifold
Dispensing station
Reaction module
Wash reservoir
Deprotection reagent reservoir
N_2 gas cylinder

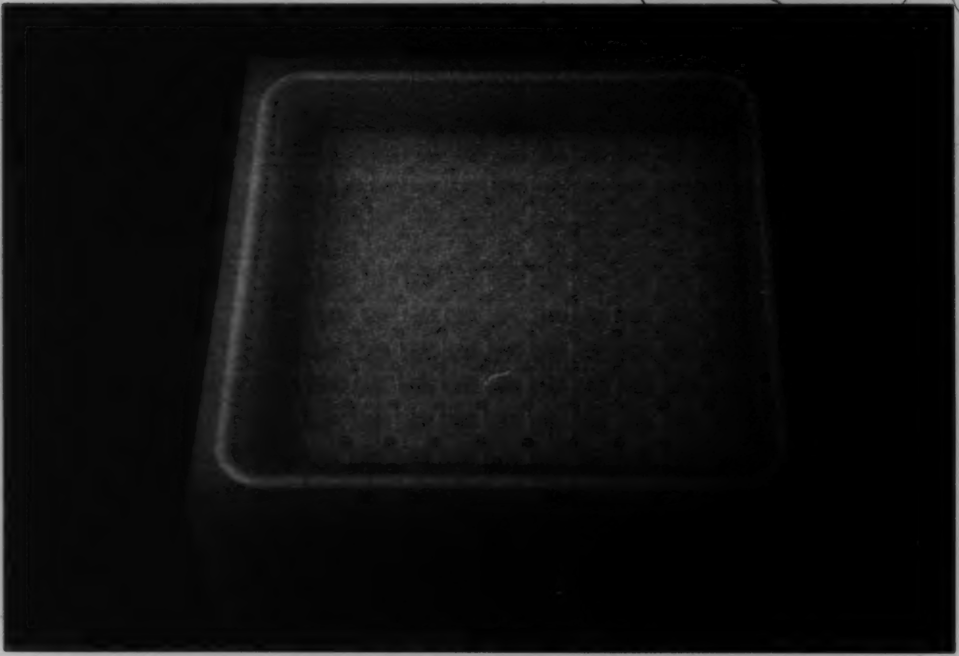
Photograph 7 Prototype reaction modules with reactor frits and insertion rod

Left: 100-reactor model

Right: 20-reactor model

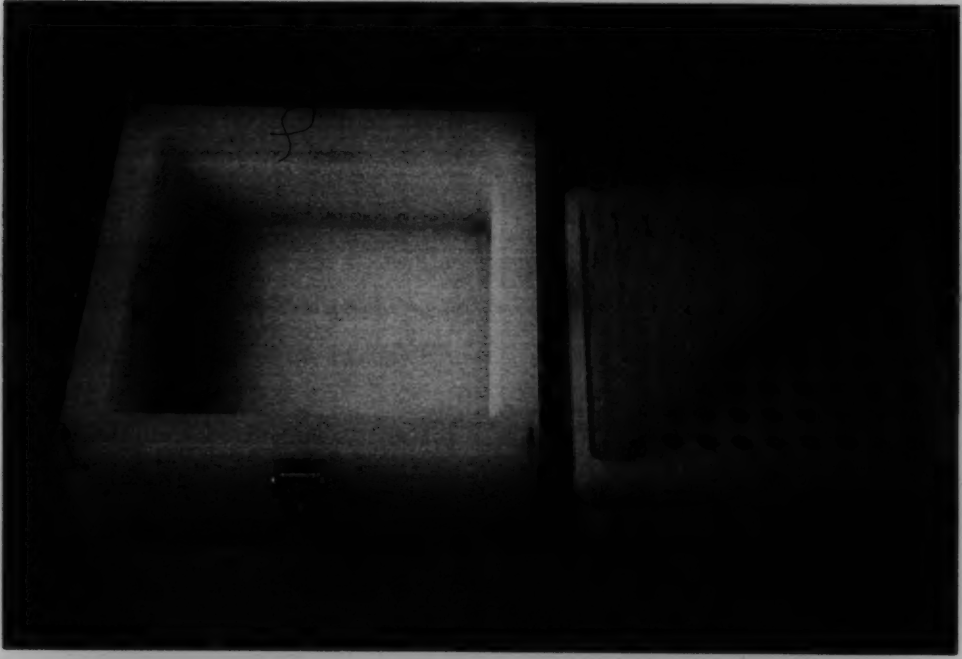


Photograph 8 Underside of 100-reactor block with recessed drainage tubes

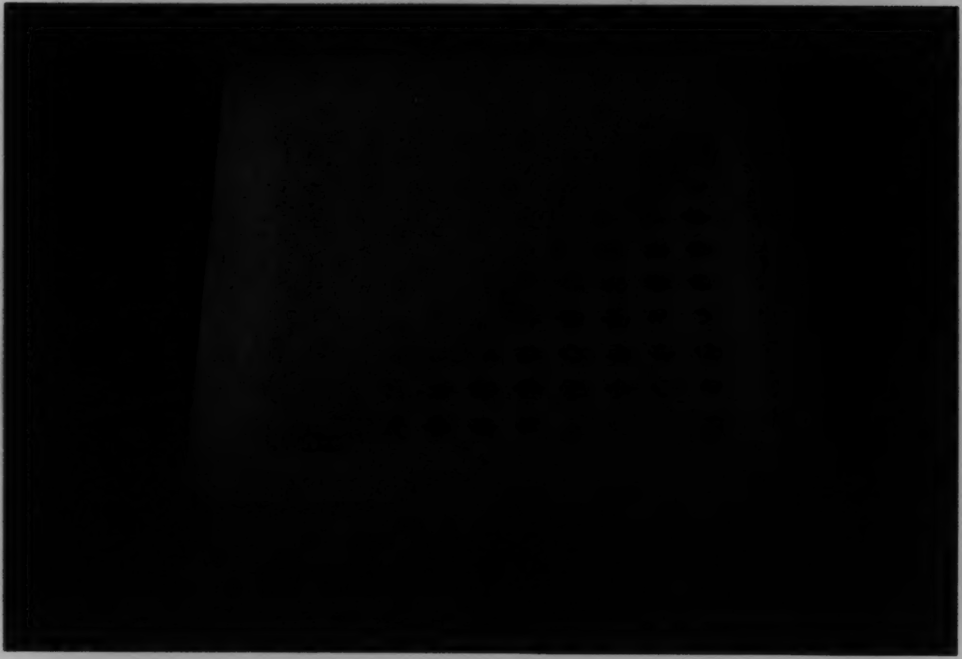


Photograph 9 Collection base of reaction module (100-reactor model)

Left: Hollow base used for synthesis, showing guide pins for error-proof assembly
Right: Cleavage collection tube rack



Photograph 10 Collection base prepared for cleavage (100-reactor model)



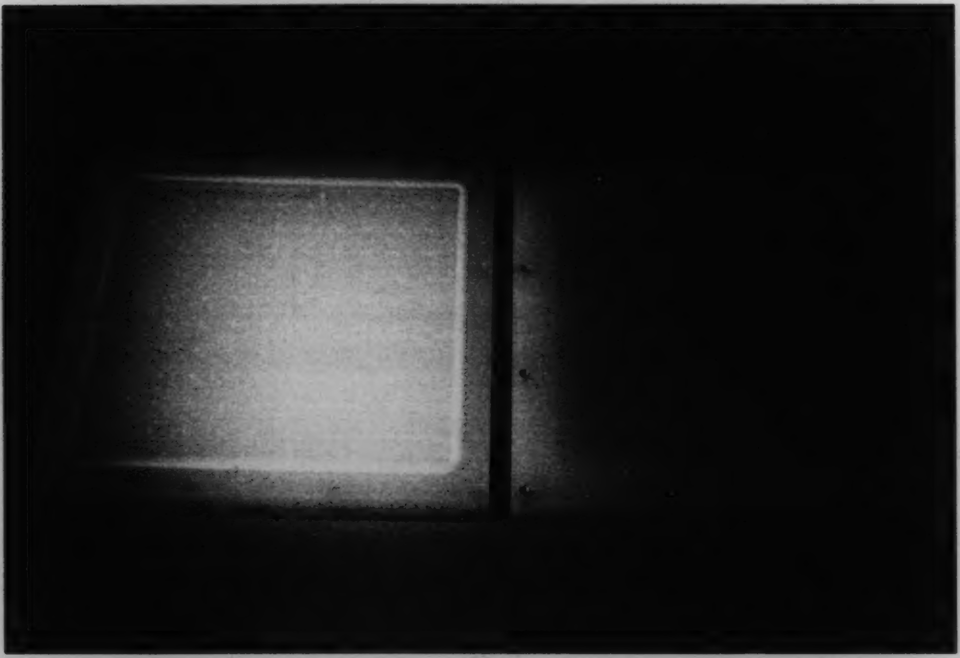
Photograph 11 Cabinet dispensing station, showing track for reaction module and positioning stop

Photograph 12 Reaction module in dispensing station (20-reactor model)

Photograph 13 Interior of disassembled dispenser module (100-reactor model)

Left: Underside of top section, showing air vent depressions

Right: Base with drainage tubes extending to interior



Photograph 14 Underside of dispenser module with recessed drainage tubes

Photograph 15 N-Evap evaporator with hollow needles for directed nitrogen gas flow

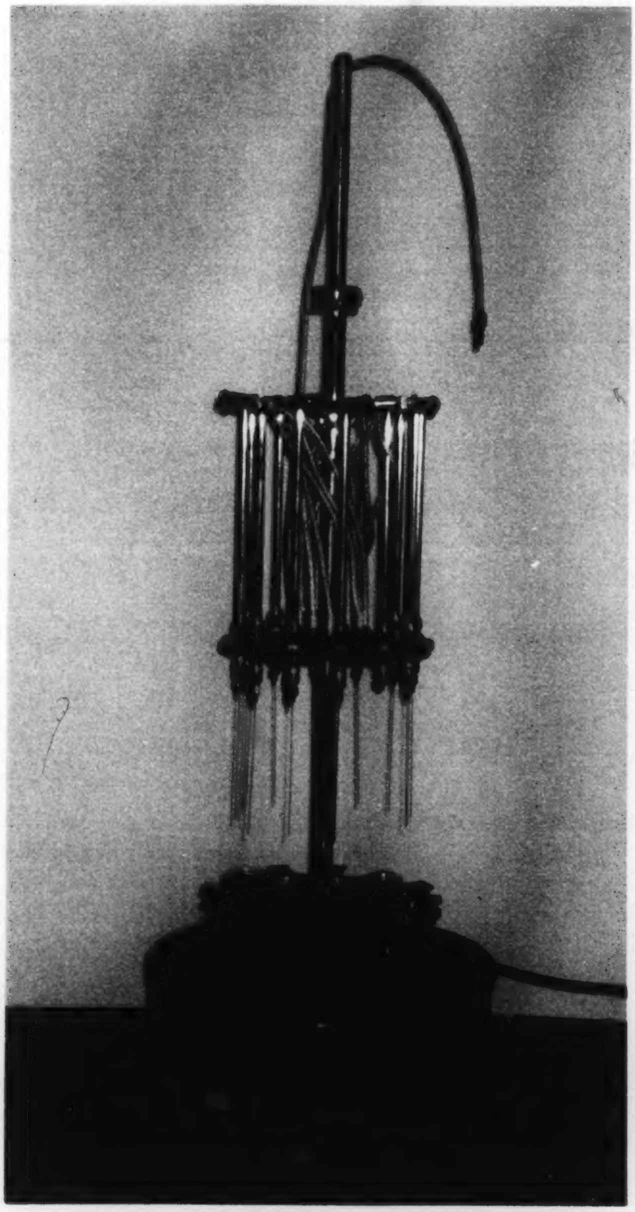
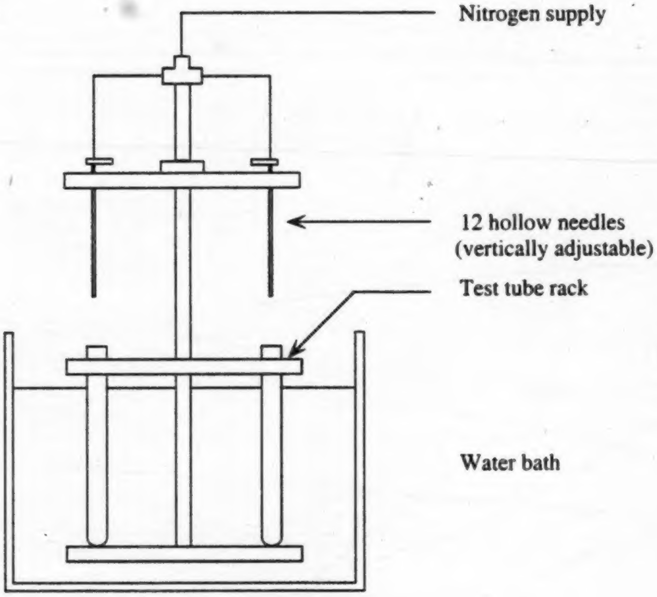


Figure 19 Schematic Representation of Evaporator


Nitrogen supply
12 hollow needles
(vertically adjustable)
Test tube rack
Water bath

3.3 Methodology

The design of the multiple peptide synthesizer prototype was predicated on the use of Fmoc chemistry, with its relatively moderate conditions for deprotection and cleavage; Fmoc protection strategy permits multiple cleavage to be performed within the open dual-purpose reaction module using TFA (as compared with the specialized apparatus required for HF cleavage in Boc chemistry). Synthesis methodology has been chosen for reliability and simplicity to allow even the most inexperienced user to proceed using a standard protocol consisting of prepared SOPs (standard operating procedures), which can be viewed in the MPS operating manual **(Appendix B)**.

NMP was initially selected as the synthesis solvent due to its enhanced ability to solvate the growing peptide chain when compared with the traditional solvent DMF, but difficulties in solubilizing certain Fmoc-amino acid derivatives (especially Asn and Gln) forced the adoption of a mixed solvent, NMP combined with DMF, to provide for elevated concentrations to assist with difficult couplings; further experience with the high cost of NMP and unreliable access to high-quality (distilled) solvent for peptide synthesis led to the final selection of DMF as sole solvent, without any noticeable effect on synthesis performance. The traditional deprotection reagent in Fmoc strategy, piperidine, was chosen for its proven record of reliability, and a double deprotection procedure using two 10-minute treatments of 20% piperidine in DMF was adopted to ensure complete removal of the N^{α}-protecting group. Multiple solvent washes between synthesis steps ensure complete reagent removal from both the dispenser module and the reaction block.

The selection of a fast and reliable activation method was vital for the multiple peptide synthesizer: in a system open to the atmosphere (and so not absolutely dry), rapid coupling is required to prevent hydrolysis of activated species, while high coupling efficiency is needed to avoid what would amount to very time-consuming monitoring of unreacted amines by a variety of methods, including the well-known quantitative ninhydrin reaction (Sarin *et al.*, 1981) or the non-invasive picrate method (Hodges and Merrifield, 1975), when performed for up to 100 reactors at a time. Although a number of Fmoc-amino acid activation methods have proven suitable for coupling in the multiple peptide synthesizer (including the use of OPf and ODHbt active esters), an *in situ* procedure generating OBt active esters from HBTU (Knorr *et al.*, 1989; Fields *et al.*, 1991) was eventually chosen as the standard activation method. Its advantages include rapid activation and coupling virtually free from racemization, high reaction efficiency by single coupling (which minimizes difficulties with residues such as Arg, Ile, Leu, Val, Asn, and Gln), the generation of harmless by-products HOBt, hexafluorophosphate, and tetramethyl urea (which is important for the open design of the reaction module), and the stability of HBTU in solution (HBTU in DMF can be stored for up to 13 weeks). The use of an *in situ* procedure also avoids the problem of declining derivative stability found with some pre-formed activated species. The method outlined in **Table 4** uses HBTU in combination with HOBt and the catalytic tertiary base DIEA in an optimal stoichiometry of 1:1:2:1 HBTU / HOBt / DIEA / Fmoc-amino acid. The conditions of coupling for the synthesizer were subject to optimization and will be discussed in the next section. Since the time of this choice of activation method, the corresponding 7-aza-based aminium salt HATU has been introduced and has demonstrated superior performance to HBTU, but this coupling reagent is not yet widely available.

Recommendations for Fmoc-compatible sidechain protection are presented in **Table 5**. The standard tBu group is normally sufficient protection for Asp and Glu as well as for Ser, Thr, and Tyr residues, and sidechain protection for Asn and Gln (which may introduce additional problems during synthesis) can usually be avoided.

The conventional Boc group is recommended for Lys, while N^{τ}-blocking of the His sidechain is achieved most reliably with the Trt group (which avoids the introduction of secondary problems caused with N^{π}-blocking by the Bum group); the Trt group is also suitable for protecting Cys residues. Pmc protection of the Arg sidechain is more expensive than the use of Mtr, but is more appropriate for the multiple peptide synthesizer due to its acid-lability: the substantial reduction in required cleavage time permits the use of a standard (and minimal) cleavage period for all reactors. No protection is necessary for the sidechains of Met and Trp in Fmoc strategy.

Although it may be possible in some cases to use aqueous TFA for final deprotection and cleavage, sidechain protection usually forces the incorporation of nucleophilic scavengers in the cleavage mixture to mop up carbonium ions and other reactive species generated during cleavage. Provision has been made in the cleavage procedure for manual addition of customized mixtures tailored to the sidechain protection of individual peptides; however, multiple cleavage using the dispensing station for reagent delivery requires an all-purpose cleavage "cocktail" of scavengers designed to be effective for a wide range of sidechain protection and cleavage problems. A modified Reagent K cleavage cocktail has been selected for use with the synthesizer, in which the scavenger phenol has been eliminated to overcome post-cleavage handling problems introduced by its relatively high viscosity (**Table 6**). EDT has been included in the cleavage cocktail to trap tBu, tBu-TFA, Pmc, and resin linker cations; thioanisole addition prevents t-butylation and oxidation of Met and increases the rate of removal of Pmc, while water is used to quench the reactivity of tBu and Pmc ions and inhibit the formation of the TFA adduct with EDT. A relatively short cleavage period of 2 hours has proven completely effective in the removal of all the recommended sidechain protection, provided that the peptide-resin within each reactor has been thoroughly dried prior to cleavage; this drying is achieved by the combination of a final DCM wash followed by a minimum 60 minute aspiration of the reaction module.

Post-cleavage peptide recovery was complicated by the sheer quantity of peptides involved. Ether precipitation (as opposed to the alternative ether extraction) was the obvious choice to minimize handling for large numbers of peptides. The conventional procedure for a single synthesis product consists of preliminary filtration of the cleavage mixture (to remove resin) before employing a rotary evaporator equipped with a CO_2/acetone cold finger to remove TFA prior to precipitation; the actual precipitation step utilizes an 8 to 10-fold volume of cold (i.e. 4° C) ether per volume of cleaved peptide solution (usually diethyl ether, although t-butylmethyl ether is also employed). At a low scale of synthesis on the multiple peptide synthesizer (i.e. no more than 0.5 mL of each cleaved peptide solution collected), ether precipitation can be carried out in the original 13 x 100 mm collection tubes which possess a working volume of approximately 5 mL, but with increasing scale there is insufficient room for ether addition at the required ratio. Considerable effort was directed at reducing solution volumes in the collection tubes using a Savant SPEEDVAC centrifugal vacuum evaporator equipped with a liquid nitrogen trap and designed to exploit centrifugal force to prevent sample loss by bumping. Despite sufficient rotor space on the high-capacity evaporator to handle as many as 100 cleaved peptide solutions simultaneously, extremely long processing times were required for even moderate reductions in solution volume within the collection tubes, which was attributed to the lack of tube rotation (and extensive exposure of liquid surface to the vacuum) found on conventional single vessel rotary evaporators. In addition, practical operation of the SPEEDVAC essentially required an additional dedicated fumehood to ventilate the extremely strong odor of EDT and thioanisole cleavage scavengers escaping during loading and unloading of collection tubes. For these reasons, volume reduction of the cleaved peptide solutions was abandoned in favor of solution transfer to larger vessels where an improved 20:1 volume ratio of ether to cleaved peptide solution could force precipitation to completion. Direct transfer by pouring (as opposed to pipetting) followed by a single minimal wash of the collection tube with neat TFA proved to be not only the simplest method but also minimized handling losses, and the choice of 50 mL conical centrifugation tubes provided ample space for vortex-washing of the crude precipitates. While these tubes permit the use of centrifugation to assist precipitate collection, gravity settling is a simpler alternative which, while potentially sacrificing some yield, avoids the costly requirement for an ether-compatible refrigerated centrifuge, and can be performed within a reasonable time period in an ice-water bath (or overnight at 4° C). Removal of supernatant and 2 serial ether washes from the precipitates was performed manually by aspiration, and the crude peptides were dried in batches of one dozen on an N-Evap evaporator (equipped with a 30° C waterbath to compensate for heat loss during evaporation) using forced nitrogen gas flow through hollow needles into the tubes to assist evaporation. Lyophilization of the crude peptides is recommended to remove volatile impurities, and pH adjustment may be performed at this time using an appropriate solvent. The conical centrifugation tubes are well suited for lyophilization: the large surface area permits easy and rapid freezing of a thin solution layer generated by manual rotation of the tube in an acetone/dry ice mixture, and the conical base prevents bumping under vacuum. The complete peptide recovery methodology is summarized in **Table 7**.

Table 4 Selected Activation Method

***In situ* generation of OBt active amino acid esters**

Prepare activated Fmoc-amino acid solutions immediately prior to use in coupling.

Reagent ratio (equivalents):

1:1:2:1 HBTU / HOBt / DIEA / Fmoc-amino acid

Reagent solutions:

0.45 M HBTU / HOBt in DMF	(stock solution)
Fmoc-amino acids dissolved in DMF	(freshly prepared)
Catalytic base DIEA	(distilled)

Table 5 Selected Fmoc-Amino Acids

Amino acid	Derivative
A	Fmoc-Ala.H_2O
C	Fmoc-Cys(Trt)
D	Fmoc-Asp(OtBu)
E	Fmoc-Glu(OtBu).H_2O
F	Fmoc-Phe
G	Fmoc-Gly
H	Fmoc-His(Trt)
I	Fmoc-Ile
K	Fmoc-Lys(Boc)
L	Fmoc-Leu
M	Fmoc-Met
N	Fmoc-Asn
P	Fmoc-Pro.H_2O
Q	Fmoc-Gln
R	Fmoc-Arg(Pmc)
S	Fmoc-Ser(tBu)
T	Fmoc-Thr(tBu)
V	Fmoc-Val
W	Fmoc-Trp
Y	Fmoc-Tyr(tBu)

Table 6 Selection of a Cleavage Reagent Cocktail

Reagent K Cocktail	Modified Reagent K Cocktail
82.5 % TFA	90 % TFA
5.0 % H_2O	5 % H_2O
5.0 % phenol	
5.0 % thioanisole	3 % thioanisole
2.5 % EDT	2 % EDT

Table 7 Selected Peptide Recovery Methodology

Cold Ether Precipitation

Perform at $\leq 4^\circ$ C (cold room or ice-water bath)

Diethyl ether : cleavage solution volume ratio:

10:1	Small-scale ($\leq$ 0.5 mL cleavage solution) Perform in 13 x 100 mm collection test tubes.
20:1	Large-scale (> 0.5 mL cleavage solution) Perform in 50 mL conical centrifuge tubes.

Collection of precipitates:

Gravity settling:	Minimum 30 - 60 minutes (or overnight).
Centrifugation:	Requires ether-compatible, refrigerated centrifuge.

Forced nitrogen gas drying of precipitates.

Lyophilization

From H_2O (may require up to 10% acetic acid to dissolve).

From NH_4HCO_3 (aq) if pH adjustment required for assay purposes.

3.4 Synthesizer Validation

Two model syntheses were chosen to validate the design of the multiple peptide synthesizer and its selected methodology: an "easy" synthesis of the octapeptide angiotensin II, and the well-known "difficult" synthesis of the C-terminal region sequence from acyl carrier protein, ACP[65-74] (sequences provided in **Table 8**, with theoretical masses of 1087.2 and 1104.2 amu, respectively). Problem syntheses can be attributed in part to steric hindrance in the coupling of amino acids such as β-branched Ile and Val, or Thr (which can be thought of as a hydroxylated version of Val), but sequence-specific difficulties also arise from poor solvation leading to H-bonding and aggregation. Intrachain H-bonding occurs most frequently at reverse turns, while interchain H-bonding and aggregation occurs with the formation of ordered structures such as α-helices and especially β-sheets in as few as 6 to 10 residues; aggregation can also occur in regions containing apolar sidechain protecting groups. Increasing solvent polarity and a change in sidechain protection may serve to disrupt aggregation. The synthesis of ACP[65-74], a sequence which contains a number of sterically hindered couplings and also promotes interchain aggregation (reducing amino group accessibility), was regarded as a test of both the efficiency of the *in situ* activation method and the degree of solvation achieved with the synthesis solvent DMF.

Both peptides were synthesized with N-terminal acetylation and C-terminal amidation, common peptide modifications to protect biological activity; since the peptide attachment to the resin was via an amide bond, normal conditions for peptide bond formation existed. In contrast, the generation of a free C-terminal peptide would have required peptide attachment via an ester bond, formerly difficult to achieve by DMAP-catalysed esterification of Fmoc-amino acid symmetric anhydrides without strictly controlled conditions to minimize racemization and dipeptide formation (and frequently circumvented by the costly purchase of resins pre-loaded with the C-terminal residue); it was not considered necessary to test the generation of peptide acids on the synthesizer since new methodology using 2,6-dichlorobenzoylchloride-catalysed esterification and longer loading times virtually eliminates these problems (Sieber, 1987). Selected methodology for N-terminal acetylation used a 1:1:20 volume ratio of acetic anhydride / DIEA / DMF.

Restricting the model syntheses to the generation of peptide amides, the performances of a selection of common amide resins in the multiple peptide synthesizer were examined. Four resins compatible with single step TFA cleavage were chosen, including two batch-mode and two continuous-flow examples (structures provided in **Figure 20**). AM (aminomethyl) and MBHA (4-methylbenzhydrylamine) batch-mode resins incorporate the Rink linker attached to a polystyrene-based support, whereas NovaSyn KR and PR resins incorporate a modified Rink linker attached to composite matrices. The loading levels of the four resins were comparable (in the range 0.41 - 0.47 mmol/g) except for the substantially lower value of 0.09 mmol/g for NovaSyn KR, and the rigidity of the latter resin provided by its Kieselguhr support also contrasted with the swelling in DMF exhibited by the remaining resins **(Table 9)**. The easy angiotensin II test synthesis was carried out on each of the resins at a 50 mg resin scale (corresponding to 0.0235, 0.0215, 0.0045 and 0.0205 mmol scale for AM, MBHA, NovaSyn KR and PR resins, with reactor solution volumes determined by swelling volumes in DMF) using less than ideal conditions of a 30 minute coupling time with 4-fold excess Fmoc-amino acid and no mixing by nitrogen bubbling to test resin performance. Although mass spectroscopy confirmed the presence of angiotensin II in all four crude products, the continuous-flow resins performed considerably better than the batch-mode resins: only minor impurities were found, whereas the AM resin crude product contained such numbers of impurities that the resin could not be considered useful under these conditions in the multiple peptide synthesizer, and even the MBHA resin produced an inferior product (**Figure 21**). The porous structure of the continuous-flow resins allows rapid diffusion and permeation of solvent and reagents to the polyamide gel where chain elongation takes place, providing for the synthesis of high purity peptides even in the absence of mixing. No sidechain-protected impurities were found by mass spectroscopy, confirming the suitability of the selected cleavage reagent cocktail and the duration of cleavage. The NovaSyn PR continuous-flow resin was chosen for further use in the multiple peptide synthesizer on the basis of purest crude product and a 4.5-fold greater loading than the Kieselguhr equivalent (NovaSyn KR).

Optimum conditions of coupling time and excess Fmoc-amino acid were determined for "easy" and "difficult" syntheses for inclusion in a standard protocol. Coupling times as rapid as 10-30 minutes are claimed for the HBTU activation method; this was investigated in the angiotensin II model synthesis using a coupling period of 10-60 minutes with no reagent limitation, i.e. a 10-fold Fmoc-amino acid excess. As seen in **Figure 22**, a reduction in coupling time from 60 to 30 minutes had little negative impact on the crude product, but further decline to 20 and 10 minutes produced increasing numbers and quantities of impurities. Consequently, only two coupling times, 30 minutes (i.e short coupling) and 60 minutes (long coupling), were retained for further use on the multiple peptide synthesizer when investigating the effect of Fmoc-amino acid excess. In the case of an easy synthesis such as angiotensin II, a short coupling time is sufficient to produce a close to pure product over a range of Fmoc-amino acid from 10-fold excess down to 5-fold excess (**Figure 23**); trace impurities increased substantially in number and quantity as regent was reduced much beyond this level. Increasing the coupling time to 60 minutes (**Figure 24**) allowed a lower Fmoc-amino acid excess to be used: even a minimal 2-fold excess produced a quite pure product. Clearly an easy synthesis on the multiple peptide synthesizer provides room for compromise between coupling time and Fmoc-amino acid excess, reducing reagent (and compensating with a prolonged coupling time) if minimal expense is the objective, or minimizing synthesis time (and compensating with a larger excess) if the objective is the most rapid peptide production. Recommended conditions in the standard protocol for an easy synthesis are 30 minute coupling at 5-fold excess. In a difficult synthesis such as ACP[65-74], this option does not exist: mass spectroscopy verified the presence of the product in all cases, but with a short coupling **(Figure 25)**, even a 10-fold amino acid excess could not reduce all impurities to trace amounts, and significant numbers and amounts of impurities appeared at even 7.5-fold excess; in a long coupling **(Figure 26)**, prolonged coupling could not completely compensate for concentration effects, since impurities became significant below a 5-fold excess. A difficult coupling requires both longer coupling and greater reagent concentration: 60 minute coupling at 10-fold excess is recommended in the standard protocol.

Reactor size in the multiple peptide synthesizer has been increased to permit a range of synthesis scale: an approximate working volume of 4 mL (which provides accommodation for mixing by nitrogen bubbling) permits a practical range of 0.005-0.2 mmol scale using the NovaSyn PR resin (corresponding to 12-490 mg resin per reactor). The possibility of a mass transfer limitation (resistance to solvent and reagent diffusion within the resin) arising with increasing scale was explored in the difficult ACP[65-74] synthesis, comparing performance under the recommended conditions (long coupling at 10-fold Fmoc-amino acid excess) at three synthesis scales: 0.02, 0.1 and 0.2 mmol **(Figure 27)**. Results confirmed the suitability of the continuous-flow NovaSyn PR resin, characterized by very high porosity and permeability towards solvent and reagents, for synthesizer use: a 10-fold increase in scale of synthesis (to

0.2 mmol) provided a product purity close to that achieved at the lowest scale (0.02 mmol), despite the absence of mixing.

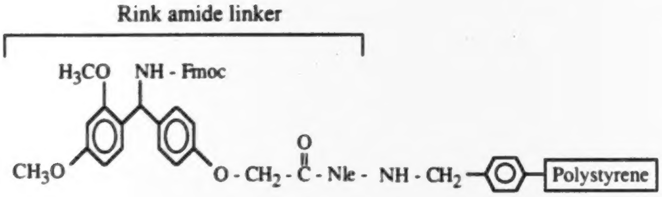
While continuous-flow resins are well-suited to the column reactor design of the multiple peptide synthesizer, their major disadvantage lies in their cost: the supported matrices required for continuous flow are considerably more expensive than the polystyrene or unsupported polyamide resins used in batch synthesis. However, the provision for mixing by nitrogen bubbling in the reactors of the multiple peptide synthesizer could provide a means for high-purity synthesis on batch-mode resins. The original angiotensin II test synthesis using 30 minute coupling with 4-fold excess Fmoc-amino acid was repeated on AM and MBHA resins with mixing during deprotection and coupling (results in **Figure 28**). A modest improvement in product quality was observed on the AM resin, while the most abundant impurity in the MBHA resin product was substantially reduced by mixing; since this synthesis was conducted at less than optimal reagent concentration, a combination of elevated reagent excess and mixing can be employed to permit the use of an inexpensive batch-mode resin in the multiple peptide synthesizer without excessive sacrifice of product quality.

Table 8 Sequences of Peptides for Model Syntheses

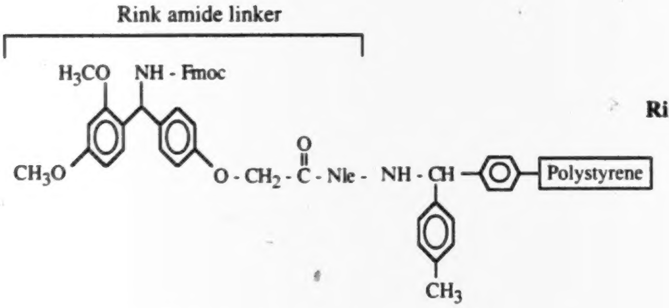
Peptide	Peptide Sequence	Synthesis Level
Angiotensin II	Ac- D R V Y I H P F -amide	Easy
ACP[65-74]	Ac- V Q A A I D Y I N G -amide	Difficult

Ac = N^{α}-acetyl; amide = C^{α}-amide.
ACP denotes acyl carrier protein.

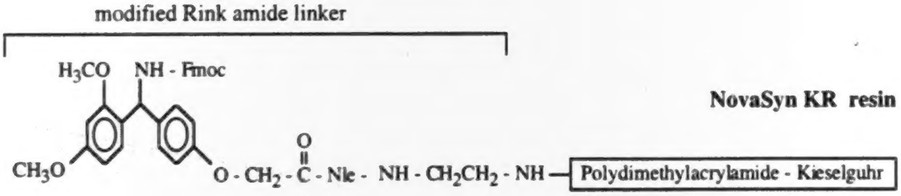
Figure 20 Structures of Selected Resins


Rink amide linker
H3CO
NH - Fmoc
CH3O
O - CH2 - C - Nle - NH - CH2
O
Polystyrene

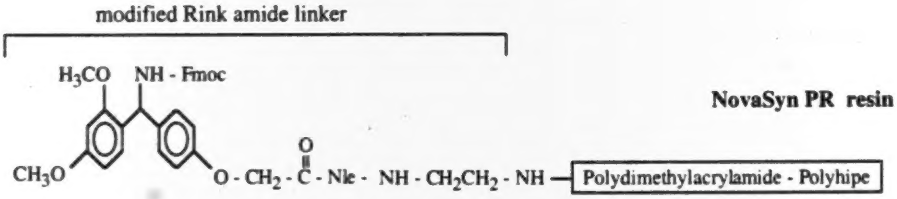
Rink amide AM resin


Rink amide linker
H3CO
NH - Fmoc
CH3O
O - CH2 - C - Nle - NH - CH
O
Polystyrene
CH3

Rink amide MBHA resin


modified Rink amide linker
H3CO
NH - Fmoc
CH3O
O - CH2 - C - Nle - NH - CH2CH2 - NH
O
Polydimethylacrylamide - Kieselguhr

NovaSyn KR resin


modified Rink amide linker
H3CO
NH - Fmoc
CH3O
O - CH2 - C - Nle - NH - CH2CH2 - NH
O
Polydimethylacrylamide - Polyhipe

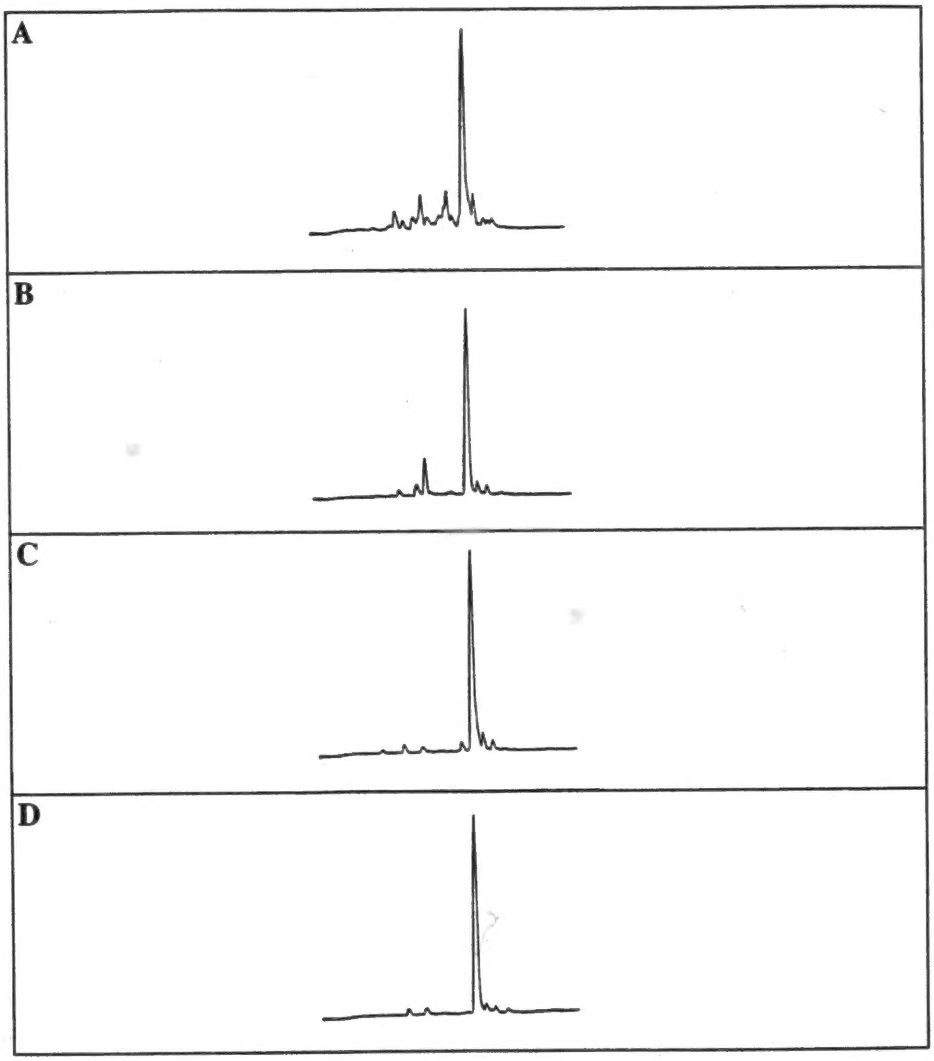
NovaSyn PR resin

Table 9 **Characteristics of Selected Resins**

Resin	Loading (mmol/g)	Swell volume in DMF (mL/g)
Rink amide AM	0.47	5.0
Rink amide MBHA	0.43	5.2
NovaSyn KR 100	0.09	3.2
NovaSyn PR 500	0.41	8.0

Figure 21 Angiotensin II test synthesis on selected resins

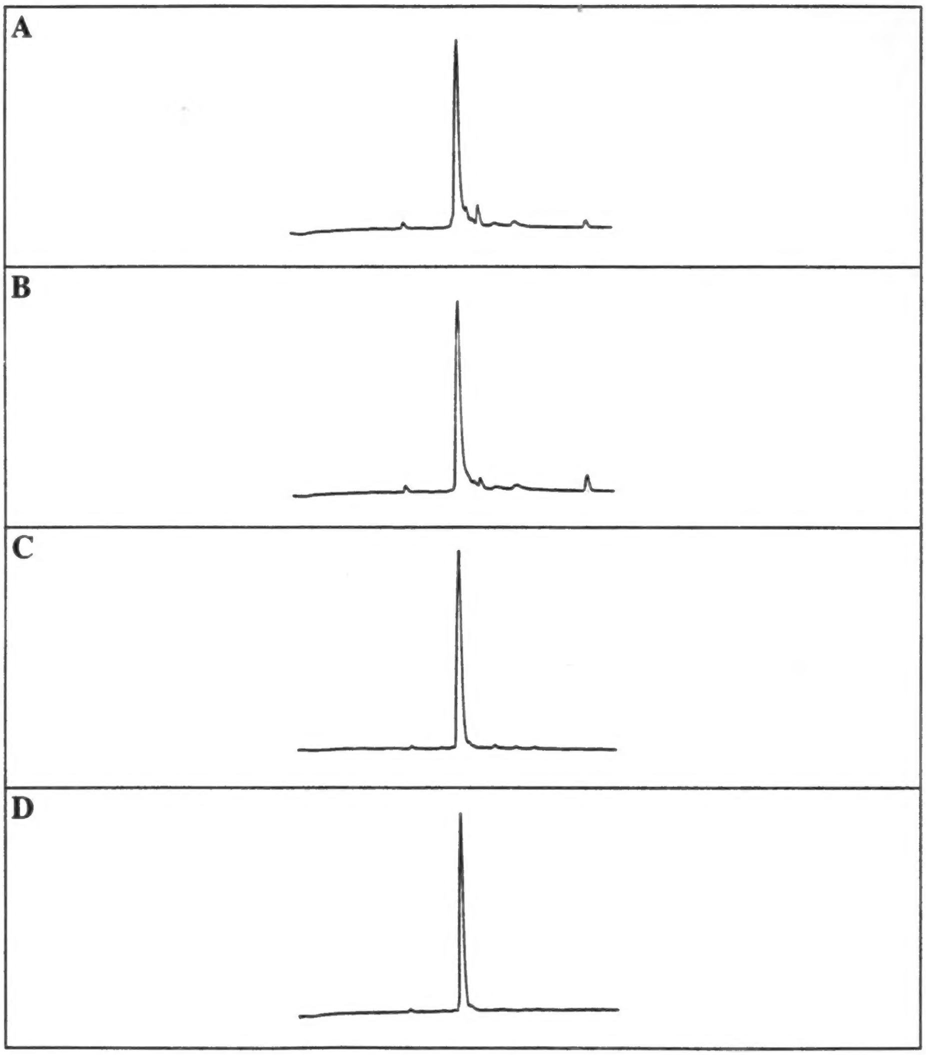
Synthesis: 30 min. coupling, 4-fold excess Fmoc-amino acid.
Panel A: AM resin.
Panel B: MBHA resin.
Panel C: NovaSyn KR resin.
Panel D: NovaSyn PR resin.



Column: Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions: Linear AB gradient (2% B/min) at a flowrate of 0.5 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Figure 22 Coupling time effect in the Angiotensin II synthesis

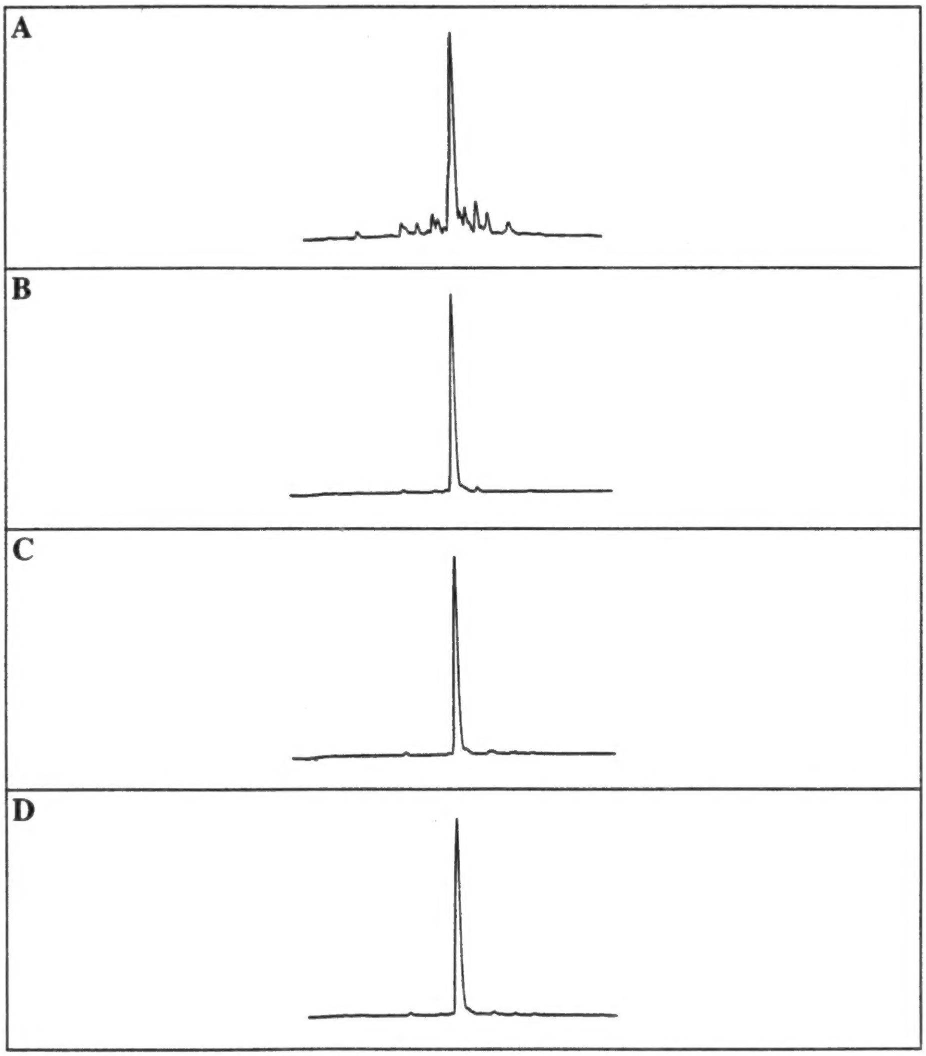
Synthesis: NovaSyn PR resin, 10-fold excess Fmoc-amino acid.
Panel A: 10 min. coupling.
Panel B: 20 min. coupling.
Panel C: 30 min. coupling.
Panel D: 60 min. coupling.



Column: Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions: Linear AB gradient (2% B/min) at a flowrate of 0.5 mL/min. Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Figure 23 Short coupling concentration effect in the Angiotensin II synthesis

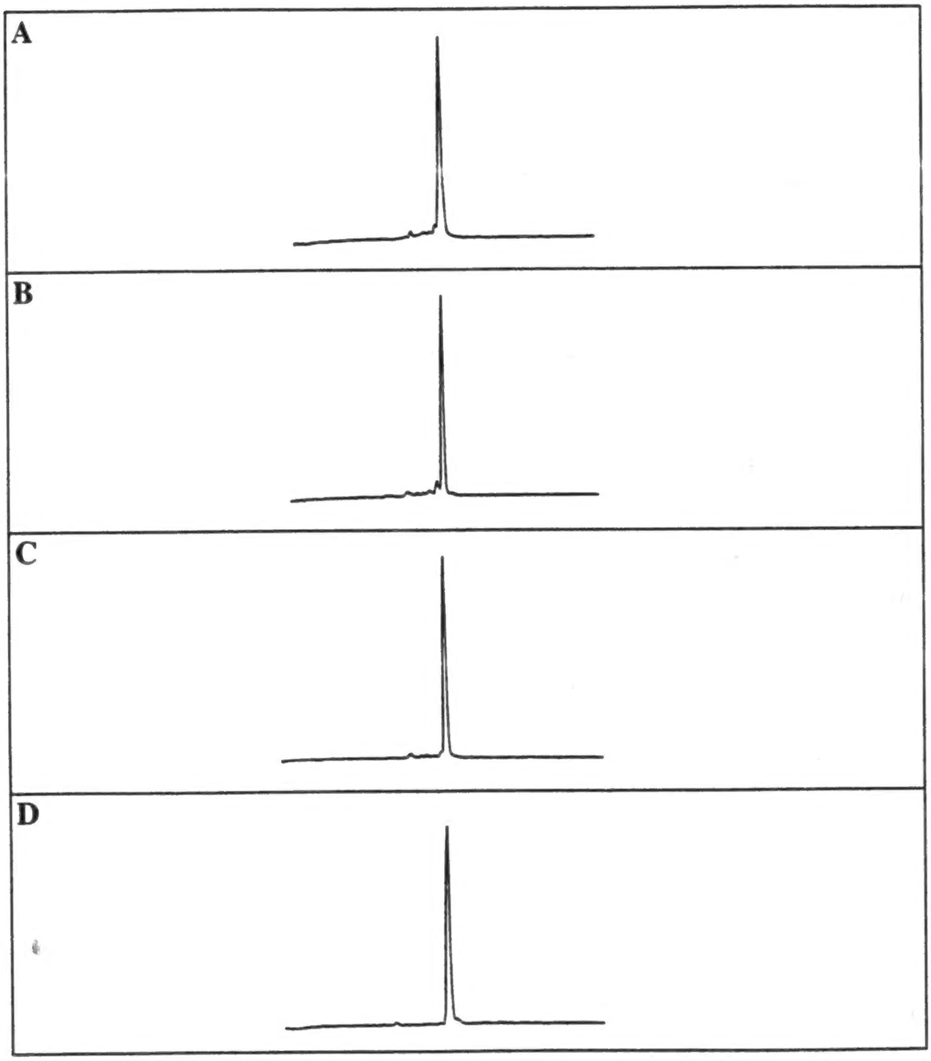
Synthesis: Short coupling refers to 30 min. coupling (on NovaSyn PR resin).
Panel A: 2-fold excess Fmoc-amino acid.
Panel B: 5-fold excess Fmoc-amino acid.
Panel C: 7.5-fold excess Fmoc-amino acid.
Panel D: 10-fold excess Fmoc-amino acid.



Column: Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions: Linear AB gradient (2% B/min) at a flowrate of 0.5 mL/min. Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Figure 24 Long coupling concentration effect in the Angiotensin II synthesis

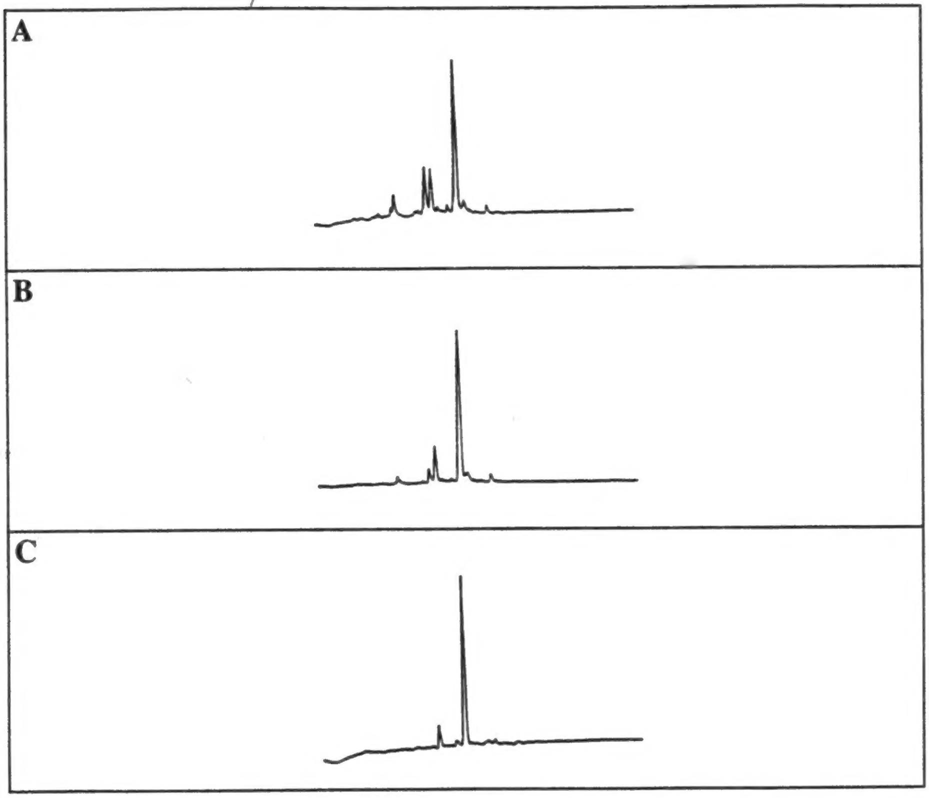
Synthesis:	Long coupling refers to 60 min. coupling (on NovaSyn PR resin).
Panel A:	2-fold excess Fmoc-amino acid.
Panel B:	5-fold excess Fmoc-amino acid.
Panel C:	7.5-fold excess Fmoc-amino acid.
Panel D:	10-fold excess Fmoc-amino acid.



Column:	Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions:	Linear AB gradient (2% B/min) at a flowrate of 0.5 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Figure 25 Short coupling concentration effect in the ACP[65-74] synthesis

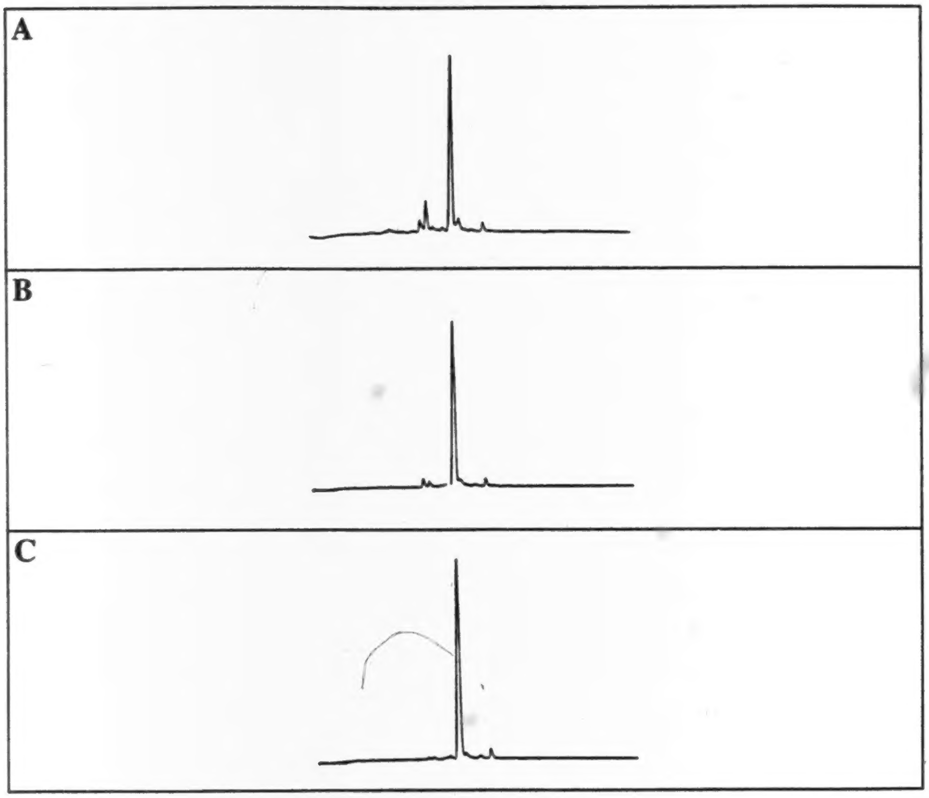
Synthesis:	Short coupling refers to 30 min. coupling (on NovaSyn PR resin).
Panel A:	5-fold excess Fmoc-amino acid.
Panel B:	7.5-fold excess Fmoc-amino acid.
Panel C:	10-fold excess Fmoc-amino acid.



Column:	Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions:	Linear AB gradient (2% B/min) at a flowrate of 0.5 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Figure 26 Long coupling concentration effect in the ACP[65-74] synthesis

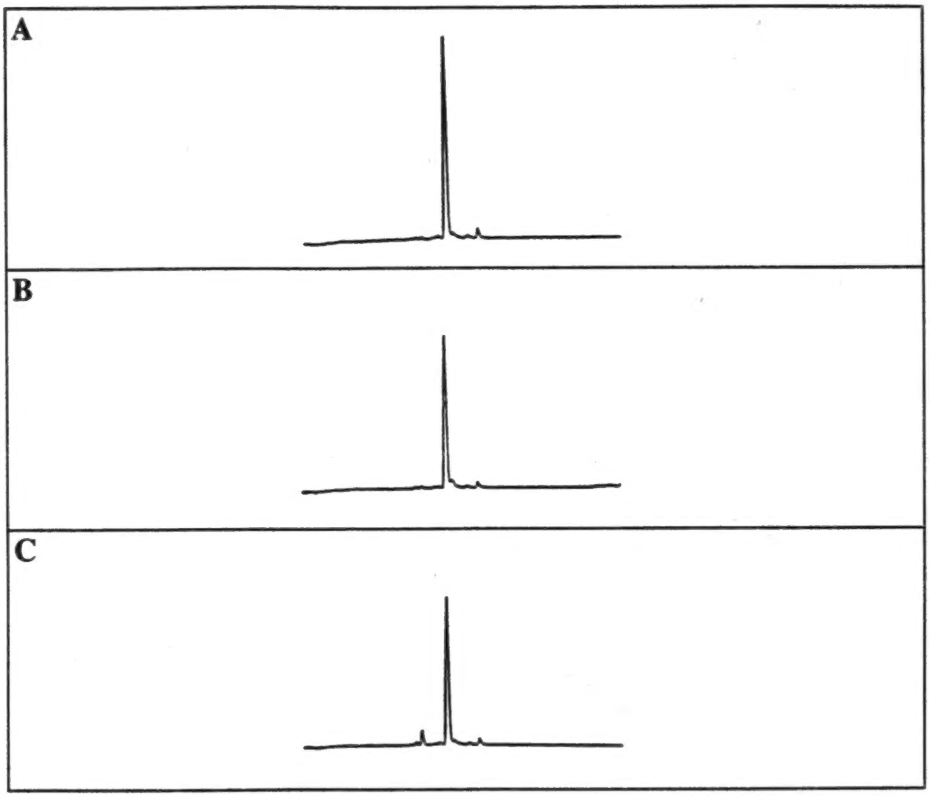
Synthesis:	Long coupling refers to 60 min. coupling (on NovaSyn PR resin).
Panel A:	5-fold excess Fmoc-amino acid.
Panel B:	7.5-fold excess Fmoc-amino acid.
Panel C:	10-fold excess Fmoc-amino acid.



Column:	Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions:	Linear AB gradient (2% B/min) at a flowrate of 0.5 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Figure 27 Effect of synthesis scale in the ACP[65-74] synthesis

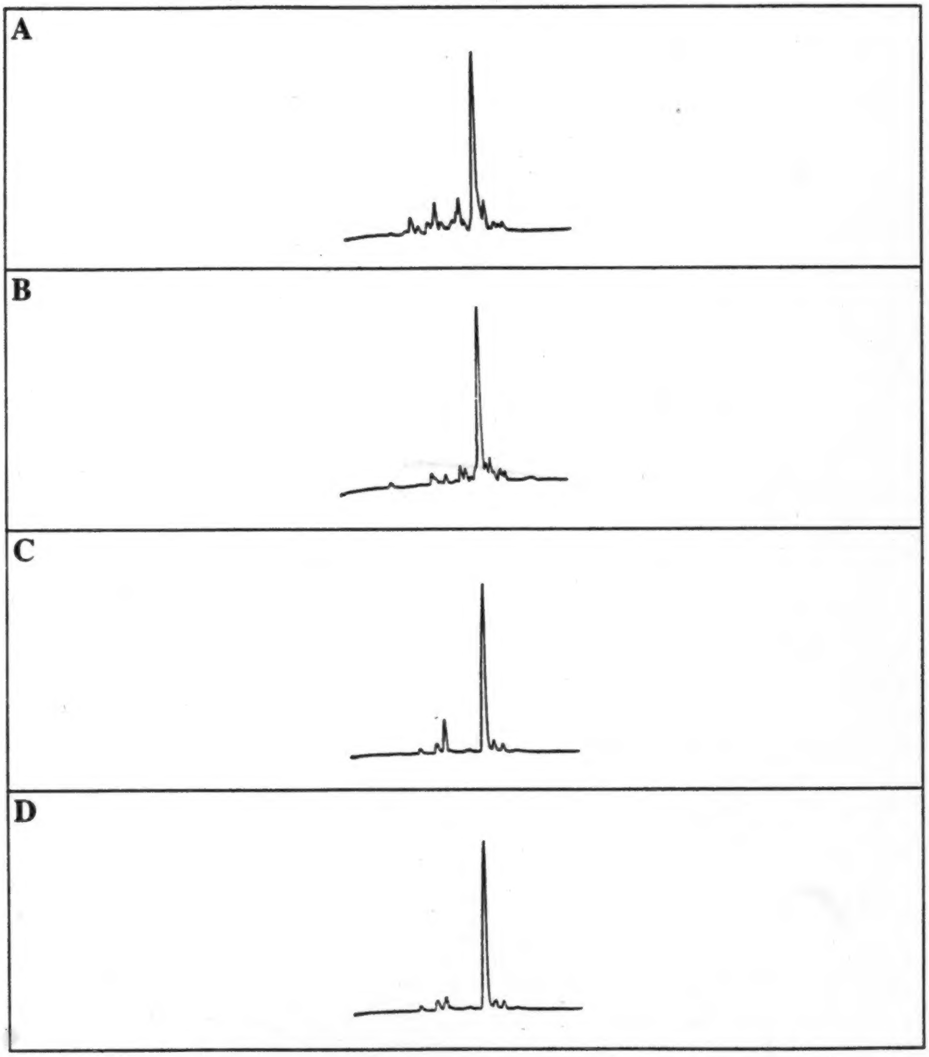
Synthesis:	Long coupling (60 min.), 10-fold excess Fmoc-amino acid (on NovaSyn PR resin).
Panel A:	0.02 mmol scale
Panel B:	0.1 mmol scale
Panel C:	0.2 mmol scale



Column:	Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions:	Linear AB gradient (2% B/min) at a flowrate of 0.5 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Figure 28 Mixing effect in the Angiotensin II synthesis

Synthesis: 30 min. coupling, 4-fold excess Fmoc-amino acid, batch resins.
Panel A: AM resin: No mixing.
Panel B: AM resin: Mixing by nitrogen bubbling.
Panel C: MBHA resin: No mixing.
Panel D: MBHA resin: Mixing by nitrogen bubbling.



Column: Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions: Linear AB gradient (2% B/min) at a flowrate of 0.5 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Chapter 4 SYNTHETIC PEPTIDE ANALOGUES (MPS APPLICATION)

4.1 Peptide Analogues and Their Uses

The generation of synthetic peptide analogues is the most common application envisaged for the multiple peptide synthesizer. Analogues with agonist or antagonist activity are useful tools in the study of the biochemistry, physiology and pharmacology of native peptide neurotransmitters, hormones, growth factors and cytokines. Small peptide mimetics of protein structure or function simplify many areas of protein research, especially molecular recognition processes. Novel analogues with pharmaceutical applications as diagnostics, therapeutics, and vaccines may be created by increasing selectivity, potency, and perhaps most importantly, stability against biodegradation. Chemical synthesis of these analogues has an advantage over biological procedures using recombinant technology - the ability to incorporate residues and structures not found in nature or subject to biological selection. The real potential of synthetic peptides lies in the creation of novel analogues with enhanced, or even entirely new, properties.

Unfortunately, while *de novo* design of peptide ligands with predicted physical, chemical and biological properties is a major goal of protein and peptide research, designing from first principles remains a complex problem. The rational approach to design continues to rely on structure-function studies, in which planned structural changes to a native sequence are introduced with the expectation of generating functional changes in the resulting analogues. These studies begin with single modifications of a native sequence to allow each change in biological activity to be assigned to an individual change in structure. For example, deletion analogues of varying length can be constructed to determine the minimum sequence for biological activity. The relative importance of individual sidechains can be assessed by residue replacement, by systematically "walking" a single alanine through the sequence to generate a series of alanine-substituted analogues (or in the event of a native alanine residue, a glycine substitution can be used); this method assumes that the sidechain deletion resulting from the substitution can assess the importance of the sidechain while having a minimum effect on peptide conformation (since alanine has the simplest C_β-containing sidechain of the amino acids). Replacement with other amino acids of differing physico-chemical properties can reveal steric, charge, hydrophobicity, and hydrogen-bonding requirements. Systematic substitution of D- for L-amino acids can demonstrate stereochemical requirements for interaction, and may also provide information on the importance of secondary structures such as α-helices and β-turns (by disrupting these structures). At a more sophisticated level, conformational constraints can be imposed on the sequence. This may be beneficial when the synthetic peptide is derived from a larger protein sequence, since the peptide may be too small to adopt a stable conformation or may lose conformational properties in solution which would have been imposed in its native protein environment. Favorable conformational constraints may produce a significant effect on even those native peptides which have evolved high biological activity despite conformational mobility. Constraints may be local (restricting the mobility of a single residue), regional (affecting a group of residues forming a secondary structural unit), or even global; examples include the addition of sterically bulky substituents adjacent to rotatable bonds (to restrict mobility), cyclic structures such as "turn mimetics" (to replace turn residues while maintaining turn geometry), and cyclization (through disulfide bond formation or amide cyclization).

The knowledge gained from the range of analogues generated in these structure-function studies can be used to identify important structural elements for biological function and molecular recognition, providing a path for the "redesign" of peptides for customized or novel activities.

4.2 Structure-Function Study of the PP-1c Binding Motif

An opportunity to demonstrate the potential of the multiple peptide synthesizer was provided by a proposed structure-function study of a novel sequence motif promoting the binding of intracellular regulatory proteins to protein phosphatase-1 in cell signalling pathways. This study seeks to define the key structural elements and determine the optimal binding motif for comparison with motifs in a variety of potential phosphatase regulatory proteins, using a set of synthetic peptide analogues generated by sequence variation of the four positions of the motif version shared by protein inhibitor I-1 and its homologue DARPP-32 **(Figure 29)**. Limited to single substitutions, and incorporating all twenty naturally occurring amino acids at each position, this study requires the synthesis of the motif-containing fragment and 76 (i.e. 4 series of 19) analogues; the effect of substitution at each position of the motif will be assessed by a competitive ^{32}P-phosphorylase *a* phosphatase radioassay, in which phosphorylase *a* serves as the phosphatase substrate and peptide analogs compete with phosphorylated I-1[9-54] to inhibit the phosphatase (Cohen *et al.*, 1988; Holmes, 1991).

Figure 29 **Generation of PP-1c binding motif analogues by sequence variation**

Motif position	**1**	**2**	**3**	**4**	
I-1 and DARPP-32 binding motif	K	I	Q	F	
Analogues					**# of analogues**
Series 1	**X**	I	Q	F	19
Series 2	K	**X**	Q	F	19
Series 3	K	I	**X**	F	19
Series 4	K	I	Q	**X**	19

X represents any one of the 20 naturally occurring amino acids.

4.3 Background

Protein phosphatase-1 (PP-1) is one of the major serine/threonine protein phosphatases of eukaryotic cells (Stralfors *et al.*, 1985), and is involved in such diverse cellular functions as glycogen metabolism, muscle contraction, neurotransmission, transcriptional splicing, and cell-cycle progression (Cohen, 1989; Shenolikar, 1994; Wera and Hemmings, 1995). In common with many protein kinases and phophatases which target serine and threonine residues (and unlike most enzymes), PP-1 displays broad substrate specificity *in vitro*. Since protein kinases and phosphatases are key components of cell signalling pathways, responsible for the reversible phosphorylation of proteins regulating most cellular processes, their broad substrate specificity permits a wide range of responses to physiological stimuli. This trait also enables cross-talk between signalling pathways (i.e. signal integration), as the range of substrates may include the protein kinases and phosphatases themselves (reviewed in Cohen, 1992).

On the other hand, broad substrate specificity necessitates strict regulation *in vivo*, and it is becoming clear that an important regulatory mechanism for a number of protein kinases and phosphatases involves compartmentalization (reviewed in Hubbard and Cohen, 1993; Faux and Scott, 1996). In this form of regulation, an enzyme does not rely on diffusion to contact its physiological substrate, but is directed through interaction with a targeting moiety or subunit to a particular subcellular environment such as an organelle, membrane, or complex. Targeting restricts enzyme location in order to confine access to a particular sustrate pool, and may even modify the catalytic and regulatory properties of protein kinases and phosphatases in order to selectively enhance specificity toward a certain substrate.

PP-1 has served as the model for developing the targeting concept. Distinct PP-1 holoenzymes appear to be associated with different cellular functions, and are composed of a 37 kDa catalytic subunit (PP-1c) complexed with one of an array of different regulatory or targeting subunits. Examples of mammalian PP-1c targeting subunits include glycogen binding protein G_M (Tang *et al.*, 1991), liver glycogen binding protein G_L (Doherty *et al.*, 1995; Moorhead *et al.*, 1995), cytosolic inhibitor proteins I-1, DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, M_r = 32,000) and I-2 (reviewed in Cohen, 1992; Hubbard and Cohen, 1993), myofibrillar binding protein M_{110} (Alessi *et al.*, 1992: Chen *et al.*, 1994; Moorhead *et al.*, 1994), retinoblastoma protein p110Rb (Durfee *et al.*, 1993), p53 binding protein p53BP2 (Helps *et al.*, 1995), and nuclear protein NIPP-1 (Jagiello *et al.*, 1995; Van Eynde *et al.*, 1995). PP-1 regulatory subunits have also been identified in yeast (reviewed in Stark, 1996).

In all, it is likely that over 100 intracellular proteins regulate PP-1c. This large number suggests the likelihood of overlapping or identical binding sites, and supporting evidence was first provided by the mutually exclusive binding of the tumour-inducing natural toxins okadaic acid and the microcystins, and the cytosolic protein inhibitors I-1 and I-2 (MacKintosh *et al.*, 1990). To date, at least a dozen PP-1c binding subunits have been shown to bind in a mutually exclusive manner. Yet initial comparison of subunit amino acid sequences showed little overall similarity. The N-terminal sequence **KIQF**, conserved in protein inhibitor I-1 and its homologue DARPP-32, was proven necessary along with a phosphorylated threonine residue for inhibition of PP-1. Disruption of the sequence, especially by loss of the Ile residue, disrupts PP-1c binding by both dephosphorylated and phosphorylated forms of the inhibitors and PP-1c inhibition by the phosphorylated forms (Desdouits *et al.*, 1995; Endo *et al.*, 1996). Since the conserved sequence is located at a distance from the phosphorylated threonine presumed to interact with the PP-1c catalytic site, it has been proposed that I-1 and DARPP-32 bind to PP-1 through two low affinity binding sites:

a putative "docking" site containing the KIQF sequence, and another site containing the phosphorylated threonine residue. A comparison of glycogen binding G_M and G_L amino acid sequences identified three short highly conserved regions, including G_M[63-86] (Doherty *et al.*, 1995). Peptides incorporating the latter sequence were shown to bind to PP-1c, disrupt its interaction with myofibrillar binding subunit M_{110}, and prevent M_{110} enhancement of PP-1c activity against the 20kDa myosin light chain subunit of smooth muscle myosin (Johnson *et al.*, 1996). Deletion analysis localized the PP-1c binding site of M_{110} to the N-terminal region (residues 1-38). These results imply mutually exclusive binding of G_M and M_{110} to PP-1c and recognition of the same binding site.

Recent co-crystallization of PP-1c with G_M[63-75] has identified structural details of G_M subunit binding (Egloff *et al.*, 1997). The G_M binding site is located opposite a shallow catalytic site channel formed at the interface of 3 β-sheets at the top of a β-sandwich. Residues [64-69] of the G_M peptide, RRVSFA, bind in an extended conformation to a hydrophobic channel formed at the bottom of the β-sandwich in the C-terminal region of PP-1c. Interactions with the peptide consist of predominantly hydrophobic contacts with Val66 and Phe68, and Ala substitutions in the peptide disrupt binding; additional interactions may occur between basic residues at the N-terminal of the peptide and a negatively-charged region of seven acidic residues surrounding the hydrophobic channel, since the acidic residues are highly conserved amongst all isoforms of PP-1 in a diverse range of species including yeast, *Drosophila*, higher plants, and mammals (Barton *et al.*, 1994). Identification of the RRVSFA binding sequence in G_M prompted re-examination of the amino acid sequences of other PP-1 regulatory subunits, despite the small degree of overall sequence similarity. Alignment of the two ankyrin repeats in p53BP2 with the second and third ankyrin repeats of M_{110} revealed the conserved sequence (R/K)VKF (similar to the RRVSFA binding sequence found in G_M) preceding the ankyrin repeats, and p53BP2[780-810] containing this sequence was shown to disrupt the interaction of both M_{110} and G_L with PP-1c. The N-terminal sequence KIQF of I-1 and DARPP-32 required for inhibition of PP-1 is also similar to the G_M binding sequence. In fact, the (R/K)(V/I)XF motif has been found in almost all known mammalian PP-1 binding proteins; in addition, a number of PP-1 binding proteins in *S. cerevisiae* contain a similar (V/I)XF sequence in which X represents a basic amino acid as found in M_{110} and p53BP2 **(Figure 30)**. This suggests that yeast proteins interacting with PP-1 probably bind to a similar hydrophobic channel .

The identification of a PP-1 binding motif reflects a growing recognition of the importance of short (4 to 6 residue) peptide sequences in governing protein-protein interactions and targeting proteins to particular subcellular environments. Examples of these sequences include the E(S/T)XV motif mediating interactions with the PDZ domain (Doyle *et al.*, 1996), the pYXXM motifs mediating interactions with SH2 domains (Zhou *et al.*, 1995), nuclear import and export sequences (Dingwall and Laskey, 1991), and the KDEL motif for targeting proteins to the lumen of the endoplasmic reticulum (Pelham, 1992). Protein sequence database searching reveals that the (R/K)(V/I)XF motif is found in 10% of proteins (Egloff *et al.*, 1997), but only 1% of these may be PP-1 binding proteins since it is estimated that around 100 PP-1 binding proteins exist in mammalian cells. While some of the motifs may be located in extracellular proteins or buried in hydrophobic cores and inaccessible for interaction on the surface, there may also be significant restrictions on the motif such as which residues can be tolerated at position X; in addition, the presence of the motif may not be sufficient in itself for binding, as demonstrated by the use of two low affinity binding sites on I-1 and DARPP-32 and evidence for the existence of a second PP-1 binding site on G_M and M_{110} (Johnson *et al.*, 1996). Structure-function studies can determine these restrictions and the optimal binding motif for comparison with motifs from suspected PP-1c regulatory proteins; the results may also prove useful in designing antagonists which, by displacing the normal targeting subunit complexed with PP-1c from a given target, may aid in the identification of intracellular targets and physiological substrates of PP-1.

Figure 30 Sequence alignment of PP-1 binding proteins in the vicinity of the (R/K)(V/I)XF motif

(Egloff *et al.*, 1997)

Mammalian	(R/K)(V/I)XF motif	Residues
G_M subunit	S G G R **R V** S **F** A D N	61 - 71
G_L subunit	K V K K **R V** S **F** A D N	57 - 67
G_L-related protein	Q A K K **R V** V **F** A D S	80 - 90
M_{110} subunit	R Q K T **K V** K **F** D D G	31 - 41
p53BP2	A H G M **R V** K **F** D D G	794 - 804
I-1	N S P R **K I** Q **F** T V P	5 - 15
DARPP-32	K D R K **K I** Q **F** S V P	4 - 14
NIPP-1	R K N S **R V** T **F** S E D	196 - 206
Splicing factor PSF	G R Q L **R V** R **F** A T H	12 - 22

S. cerevisiae	Putative PP-1 binding motif	Residues
GAC1	S P E **K** N **V** R **F** A I E	66 - 76
PIG2	S S G **K** S **V** R **F** A A H	50 - 60
GIP2	I R S **K** S **V** H **F** D Q A	217 - 227
YIL045W	Q R S **K** S **V** H **F** D R V	193 - 203
	V F V **K** N **I** Y **F** S N A	412 - 422
REG1	T K N **R** H **I H F** N D R	461 - 471
REG2	P R E **R** H **I K F** N D N	164 - 174
SCD5	F K S **K** K **V R F** S E H	270 - 280
GIP1	W N L **K** F **I** P **F** N N L	180 - 190
	K K K **R** C **V** N **F** R N K	441 - 451

4.4 Analogue Design and Synthesis

Preliminary work by Dr. Charles Holmes (University of Alberta), instigator of the PP-1c binding motif structure-function study, has focussed on protein inhibitor I-1 and deletion analogues of the fully active I-1[9-54] fragment **(Figure 31)**. The importance of the second position in the motif is reflected in the reduced inhibitory activity against PP-1c of N-terminal deletion analogues: while loss of the first residue (Lys) brought about a relatively modest 5-fold decrease in activity, the combined loss of the first and second residues (Lys and Ile) caused a 1000-fold decline. Comparison of the activities of a limited number of second position substitution analogues suggests poor tolerance for residue variation at this position: although substitution for Ile of a similar hydrophobe, Leu, resulted in only a 2.5-fold decrease in activity, substitution of a larger hydrophobe such as Phe brought about a 100-fold drop, and the presence of non-hydrophobic residues (such as Gly, Lys, or Glu) in this position produced even more drastic reductions in activity. The I-1 and DARPP-32 version of the binding motif is of special interest since it is considered to act as a low affinity "docking" site for PP-1 binding, lacking any regulatory function which would impose restrictions on the motif beyond binding requirements. Instead, a second low-affinity site containing a phosphorylated Thr residue is presumed to interact with the catalytic site of PP-1c and to serve a regulatory role (since the inhibitor must be phosphorylated by PKA at the Thr35 residue in order to inhibit PP-1c); binding at both sites is required for high affinity interaction and inhibition of the phosphatase by the inhibitor. This is in contrast to the version of the binding motif found in G_M (RVSF), which appears to combine both binding and regulatory functions at the same site (since PKA phosphorylation of Ser67 in the third position of the motif prevents the interaction of G_M and PP-1c, presumably by direct competition for the binding motif). The same mechanism may also be employed by NIPP-1, which ceases to inhibit PP-1c once phosphorylated by

PKA and/or casein kinase 2; NIPP-1 has one consensus PKA phosphorylation site immediately N-terminal to the motif and two casein kinase 2 consensus phosphorylation sites, one within the motif and the other immediately C-terminal to the fourth position Phe residue (Van Eynde *et al.*, 1995).

Initially, an 8-residue peptide length was chosen for the analogue series, incorporating residues on either side of the binding sequence. Since I-1 and DARPP-32 differ by three residues within the chosen 8-residue sequence, both I-1[7-14] and DARPP-32[6-13] alternatives were synthesized as N-acetylated peptide amides for consideration as the analogue template (sequences and RP-HPLC chromatograms provided in **Figure 32**); mass spectroscopy provided excellent agreement between calculated and experimental masses (1029.3 vs 1029.9 amu for I-1[7-14], and 1046.3 vs 1047.0 amu for DARPP-32[6-13]. Little difference between the two peptides was observed in terms of synthesis performance (yield and purity), solubility (a major limitation in the competitive assay), or PP-1c inhibitory activity, despite the presence of a second Lys residue expected to improve DARPP-32[6-13] solubility; consequently, selection of the I-1 template was based on maintaining consistency with the previous work. In an attempt to increase poor solubility, template length was increased to incorporate polar and ionizable residues Ser6 and Glu18 in a 13-residue peptide and His20 in a 15-residue peptide (sequences and RP-HPLC chromatograms provided in **Figure 33**); mass spectroscopy provided excellent agreement between calculated and experimental masses (1029.3 vs 1029.9 amu for I-1[7-14], 1568.9 vs 1569.0 amu for I-1[6-18], and 1803.1 vs 1803.4 amu for DARPP-32[6-13]). Although the 13-residue peptide exhibited only a limited improvement in solubility (along with a small increase in synthesis purity), the longer template was chosen to allow comparison with the 13-residue G_M peptide co-crystallized with PP-1c by Egloff *et al.*, 1997 .

I-1[6-18] analogues of the binding motif were generated by single substitutions of all 20 naturally-occurring amino acids in positions 1 through 4 of the motif, denoted by position and substitution (i.e. 1A refers to the Ala-substituted position 1 analogue, AIQF). The N^{α}-acetylated peptide amides were synthesized simultaneously on the 100-reactor model of the multiple peptide synthesizer at 0.02 mmol scale, following the standard protocol for the synthesizer: synthesis on continuous flow NovaSyn PR resin; deprotection by 20% piperidine in DMF; Fmoc-amino acid sidechain protecting groups of OtBu (Asp, Glu), tBu (Ser, Thr, Tyr), Boc (Lys), Trt (Cys, His), and Pmc (Arg); *in situ* activation of Fmoc-amino acids to generate OBt esters from HBTU; N-terminal acetylation using excess acetic anhydride; cleavage by modified Reagent K; and cold ether precipitation of peptides. A 10-fold excess reagent concentration was used with a 60 minute coupling time for incorporating the most difficult residues and ensuring a sufficient level of purity to allow preliminary screening in the competition assay without peptide purification. The crude analogues were analyzed by RP-HPLC, mass spectroscopy, and amino acid analysis, and the results are summarized in **Table 10**. Both purity and yield were lower than expected (averaging 70% and 60%, respectively) given the excellent test synthesis of the template, although analogue chromatograms presented in **Figure 34** reveal a single major peak in virtually every case, corresponding to the expected peptide mass. The Cys and Asn analogues at each of the four positions of the motif proved difficult to synthesize, the former containing both oxidized and reduced forms (as expected) and the latter contaminated by the Asn-deletion peptide in all but the 1-position. The Gln-deletion peptide constituted the major (and consistent) impurity in this set of syntheses, especially in the case of the Cys analogues as well as the Leu and Asn analogues in the 1 position. Other contaminants included the occasional incomplete sidechain deprotection peptide (Pmc and tBu groups remaining) and a very minor but consistent non-acetylated product and a number of

Lys-deletion peptides in the three-position analogues. The degree of purity achieved in the synthesis appeared to be affected by the position of substitution, as shown in **Figure 35**: the majority of position 2 and position 3 analogues were synthesized in the 70-89% range, position 1 analogues were obtained mainly in the 70-79% purity range, and position 4 analogues were the most difficult to synthesize, reflecting purities mainly in the 60-69% range. The crude peptides were deemed sufficiently pure for preliminary screening by competition assay, reducing the future task of purification to the most promising candidates identified by this on-going screening.

Figure 31 Inhibitory activity of I-1 peptide sequences against PP-1

(Personal communication, Dr. Charles Holmes, University of Alberta)

I-1 peptide sequence	Relative activity (%)
9 35 41 54	
K I Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L T S D Q S S P E V D E D R	100
K I Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	24
I Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	5
Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	0.006
F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	0.004
T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	0.003
K L Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	9.0
K C Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	3.0
K A Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	1.4
K T Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	0.400
K F Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	0.250
K G Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	0.050
K K Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	0.030
K E Q F T V P L L E P H L D P E A A E Q I R R R R P T* P A T L V L	0.015

* refers to phosphorylated residue. Variation in PP-1c binding sequence shown in box.

Activity determined for N-acetylated, C-amidated peptides using ^{32}P-phosphorylase *a* phosphatase radioassay (Cohen *et al.* , 1988; Holmes, 1991).

Figure 32 **I-1 and DARPP-32 potential analogue templates**

Panel A: Sequences of motif-containing synthetic octapeptides I-1[7-14] and DARPP-32[6-13]
Panel B: Analytical RP-HPLC of I-1[7-14]
Panel C: Analytical RP-HPLC of DARPP-32[6-13]

Column: Zorbax 300SB C8 (250 x 4.6 mm)
Conditions: Linear AB gradient (1% B/min) at a flowrate of 1 mL/min,
Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

A

I-1[7-14]	Ac-	**P**	**R**	K	I	Q	F	**T**	V	-amide
DARPP-32[6-13]	Ac-	**R**	**K**	K	I	Q	F	**S**	V	-amide

PP-1c binding motif shown in box
Ac = N^{α}-acetyl; amide = C^{α}-amide

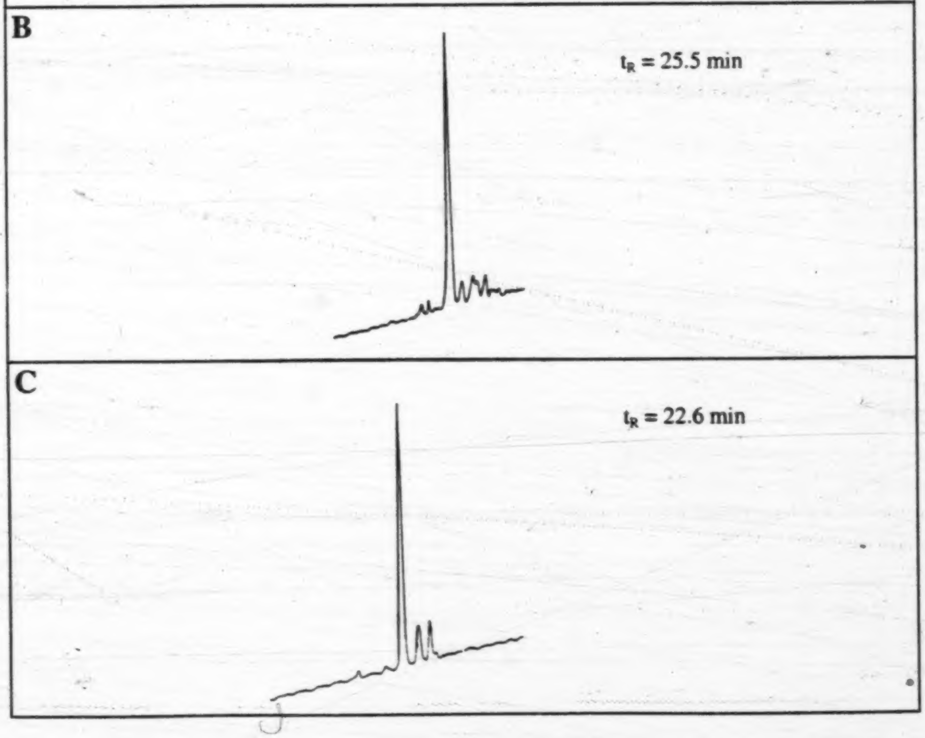


Figure 33 I-1 analogue template length

Panel A:	Sequences of 8-, 13-, and 15-residue I-1 potential analogue templates
Panel B:	Analytical RP-HPLC of I-1[7-14]
Panel C:	Analytical RP-HPLC of I-1[6-18]
Panel D:	Analytical RP-HPLC of I-1[6-20]
Column:	Zorbax 300SB C8 (250 x 4.6 mm)
Conditions:	Linear AB gradient (1% B/min) at a flowrate of 1 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

A

I-1[7-14]	Ac-		P	R	K	I	Q	F	T	V							-amide
I-1[6-18]	Ac-	**S**	P	R	K	I	Q	F	T	V	P	L	L	**E**			-amide
I-1[6-20]	Ac-	**S**	P	K	K	I	Q	F	T	V	P	L	L	**E**	P	**H**	-amide

PP-1c binding motif shown in box.
Residues for improving solubility shown in bold.

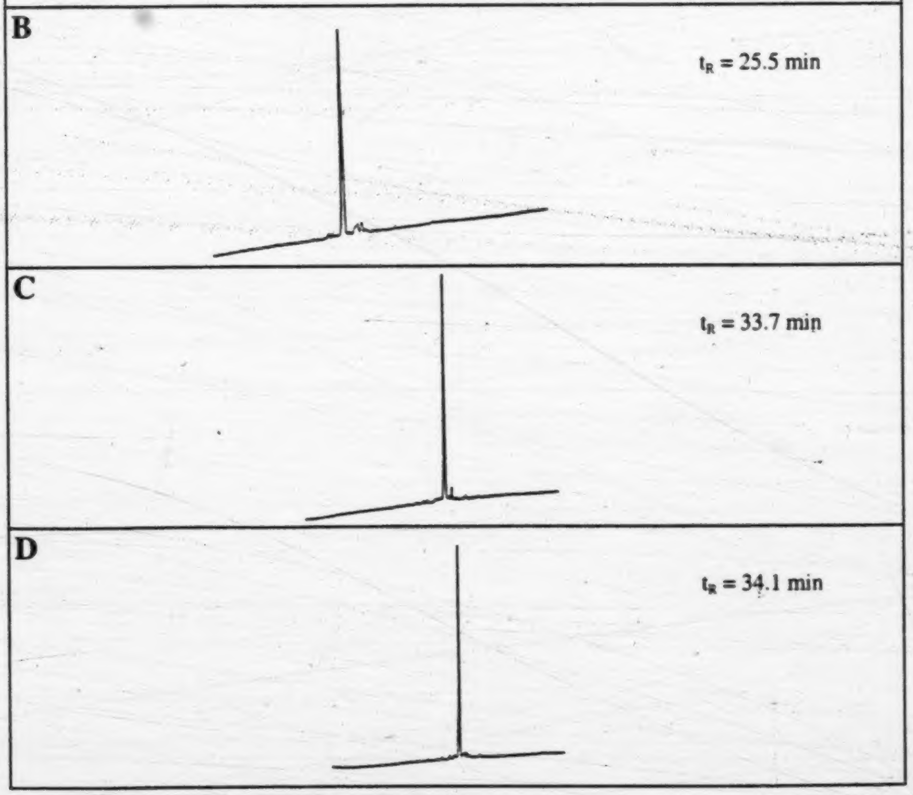


Table 10 KIQF Analogue Analytical Data

Analogue	Sequence	HPLC		Mass (amu)		Contaminants	Yield
		t_R (min)	Purity (%)	Calculated	Experimental		(% theoretical)
1A	AIQF	35.6	72	1511.8	1512.0	Q, F deletions	67
1C	CIQF	36.3	45	1543.9	1543.7	Q deletion	35
1D	DIQF	35.1	73	1555.8	1555.6	Q deletion	67
1E	EIQF	35.4	71	1569.8	1569.8	Q deletion	65
1F	FIQF	39.0	66	1587.9	1587.6	Q deletion	50
1G	GIQF	35.2	73	1497.8	1497.8	F deletion	66
1H	HIQF	33.8	83	1577.8	1578.0	Q deletion	74
1I	IIQF	38.3	73	1553.9	1554.2	Q deletion	71
1K	KIQF	33.7	82	1568.9	1568.8	Q deletion	79
1L	LIQF	38.9	38	1553.9	1554.2	Q deletion	37
1M	MIQF	37.7	68	1571.9	1572.0	Q deletion	55
1N	NIQF	34.5	25	1554.8	1554.4	Q deletion	17
1P	PIQF	35.4	71	1537.8	1538.0	Q deletion	61
1Q	QIQF	34.7	70	1568.8	1568.4	Q deletion	58
1R	RIQF	34.0	78	1596.9	1596.4	Q deletion	68
1S	SIQF	34.9	74	1527.8	1527.6	Q deletion	65
1T	TIQF	35.3	65	1541.8	1541.6	Q deletion	62
1V	VIQF	37.0	51	1539.8	1540.0	Q deletion	43
1W	WIQF	40.0	69	1626.9	1627.0	Q deletion	56
1Y	YIQF	36.8	55	1603.9	1603.6	Q deletion	37

Table 10 KIQF Analogue Analytical Data (cont'd)

Analogue	Sequence	HPLC		Mass (amu)		Contaminants	Yield
		t_R (min)	Purity (%)	Calculated	Experimental		(% theoretical)
2A	KAQF	31.3	89	1526.8	1527.0		76
2C	KCQF	31.8	30	1558.9	1559.0	Q deletion	19
2D	KDQF	31.3	79	1570.8	1570.5	Q deletion	69
2E	KEQF	31.2	89	1584.8	1585.0	Q deletion	85
2F	KFQF	35.0	84	1602.9	1602.8	Q deletion	73
2G	KGQF	31.0	79	1512.8	1512.8	Q deletion	65
2H	KHQF	30.4	93	1592.9	1592.7	Q, H deletions	81
2K	KKQF	29.8	90	1583.9	1583.2	Q deletion	88
2L	KLQF	34.2	74	1568.9	1568.8	Q deletion	72
2M	KMQF	33.4	73	1586.9	1586.9	Q deletion	60
2N	KNQF	32.4	21	1569.8	1569.8	Q, N deletions	20
2P	KPQF	31.8	87	1552.8	1553.0	Q deletion	77
2Q	KQQF	30.7	84	1583.9	1583.9	Q deletion	76
2R	KRQF	30.0	92	1611.9	1612.2	Q deletion	87
2S	KSQF	30.9	76	1542.8	1543.0	Q deletion	71
2T	KTQF	31.2	76	1556.8	1557.1	Q deletion	74
2V	KVQF	32.7	75	1554.9	1555.2	Q deletion	61
2W	KWQF	35.6	58	1641.9	1642.0	Q deletion	53
2Y	KYQF	33.0	72	1618.9	1618.8	Retained Pmc group Q deletion	61

Table 10 KIQF Analogue Analytical Data (cont'd)

Analogue	Sequence	HPLC		Mass (amu)		Contaminants	Yield
		t_R (min)	Purity (%)	Calculated	Experimental		(% theoretical)
3A	KIAF	34.4	80	1511.8	1511.5	No N-acetyl	65
3C	KICF	35.1	40	1543.9	1543.7	No N-acetyl	32
3D	KIDF	34.0	78	1555.8	1556.1	No N-acetyl	68
3E	KIEF	34.4	82	1569.9	1569.8	No N-acetyl	68
3F	KIFF	37.4	87	1587.9	1587.8	No N-acetyl	60
3G	KIGF	33.8	86	1497.8	1498.0	No N-acetyl	69
3H	KIHF	33.1	74	1577.9	1578.1	No N-acetyl H+K deletion	60
3I	KIIF	36.3	87	1553.9	1554.2	No N-acetyl	76
3K	KIKF	32.7	84	1568.9	1569.1	N-acetyl, K deletion	75
3L	KILF	36.9	77	1553.9	1554.2	No N-acetyl	71
3M	KIMF	36.0	87	1571.9	1572.0	No N-acetyl	62
3N	KINF	33.7	21	1554.9	1555.2	N-acetyl, N deletion	18
3P	KIPF	34.7	82	1537.9	1538.1	K deletion	72
3R	KIRF	33.2	84	1596.9	1596.9	N-acetyl, K deletion	69
3S	KISF	34.0	78	1527.8	1527.6	No N-acetyl	70
3T	KITF	34.2	83	1541.9	1542.0	No N-acetyl	70
3V	KIVF	35.3	81	1539.9	1539.8	N-acetyl, K deletion	74
3W	KIWF	38.1	73	1627.0	1627.1	N-acetyl, K deletion	55
3Y	KIYF	35.4	89	1603.9	1603.4	No N-acetyl	67

Table 10 KIQF Analogue Analytical Data (cont'd)

Analogue	Sequence	HPLC		Mass (amu)		Contaminants	Yield
		t_R (min)	Purity (%)	Calculated	Experimental		(% theoretical)
4A	KIQA	28.8	70	1492.8	1492.7	Q deletion	62
4C	KIQC	29.0	27	1524.8	1524.9	Q deletion	21
4D	KIQD	28.8	68	1536.8	1537.1	Q deletion	66
4E	KIQE	28.8	80	1550.8	1550.8	Q deletion	71
4G	KIQG	27.7	61	1478.8	1479.1	Q deletion	54
4H	KIQH	26.4	84	1558.8	1558.8	Q deletion	56
4I	KIQI	32.6	75	1534.9	1535.2	Retained tBu group Q deletion	66
4K	KIQK	26.1	64	1549.9	1550.0	Q deletion	57
4L	KIQL	32.6	68	1534.9	1535.2	Retained tBu group Q deletion	55
4M	KIQM	31.5	64	1552.9	1552.7	Q deletion	59
4N	KIQN	29.7	20	1535.8	1535.7	N, Q+E deletions	17
4P	KIQP	28.5	64	1518.8	1519.1	Q deletion	63
4Q	KIQQ	28.0	67	1549.8	1550.0	Q deletion	52
4R	KIQR	26.3	68	1577.9	1577.8	Q deletion	63
4S	KIQS	27.8	71	1508.8	1508.6	Retained tBu group Q deletion	61
4T	KIQT	28.2	62	1522.8	1523.0	Q deletion	52
4V	KIQV	31.0	66	1520.8	1521.3	Q deletion	52
4W	KIQW	34.3	69	1607.9	1608.1	Q deletion	51
4Y	KIQY	30.5	75	1584.9	1585.2	Q deletion	61

Figure 34 RP-HPLC chromatograms of I-1[6-18] synthetic peptide analogues

Column: Zorbax 300SB C8 (250 x 4.6 mm)

Conditions: Linear AB gradient (1% B/min) at a flowrate of 1 mL/min,
Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

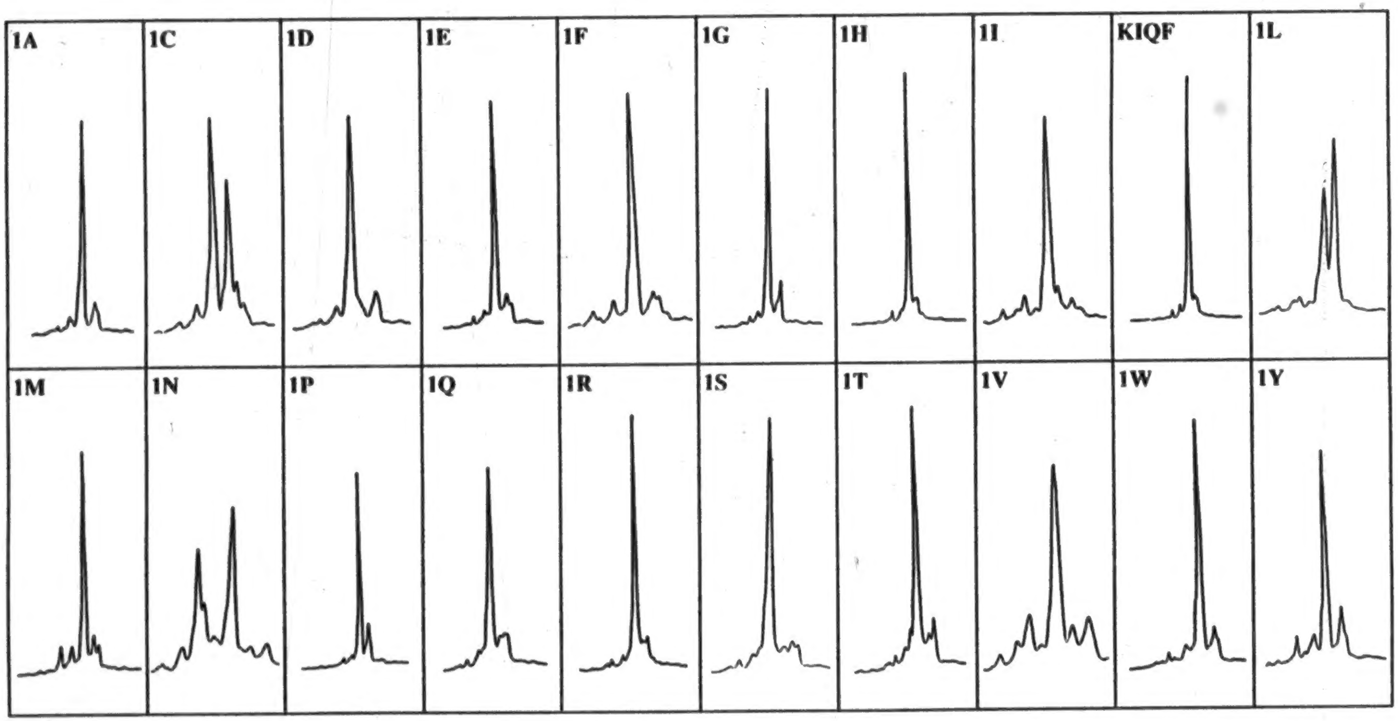


Figure 34 RP-HPLC chromatograms of I-1[6-18] synthetic peptide analogues (cont'd)

Column: Zorbax 300SB C8 (250 x 4.6 mm)
Conditions: Linear AB gradient (1% B/min) at a flowrate of 1 mL/min,
Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

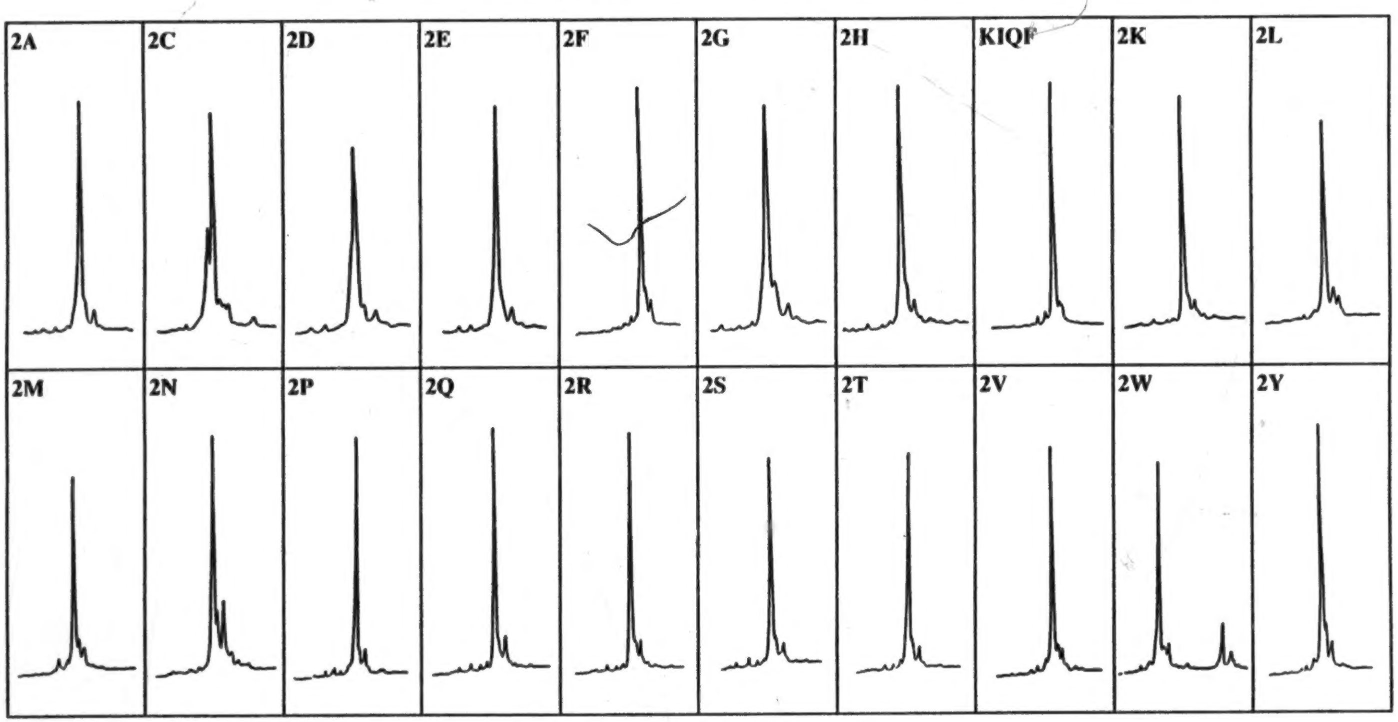


Figure 34 RP-HPLC chromatograms of I-1[6-18] synthetic peptide analogues (cont'd)

Column: Zorbax 300SB C8 (250 x 4.6 mm)
Conditions: Linear AB gradient (1% B/min) at a flowrate of 1 mL/min,
Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

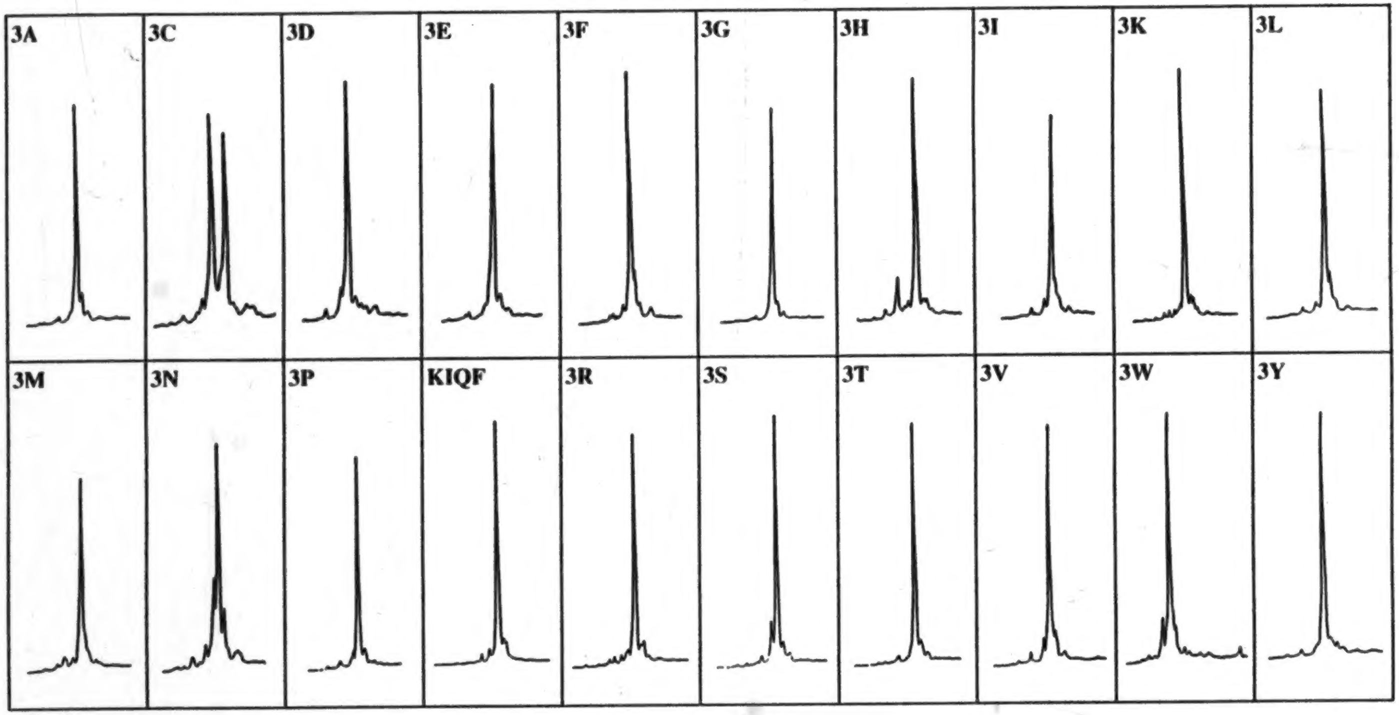


Figure 34 RP-HPLC chromatograms of I-1[6-18] synthetic peptide analogues (cont'd)

Column: Zorbax 300SB C8 (250 x 4.6 mm)
Conditions: Linear AB gradient (1% B/min) at a flowrate of 1 mL/min,
Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

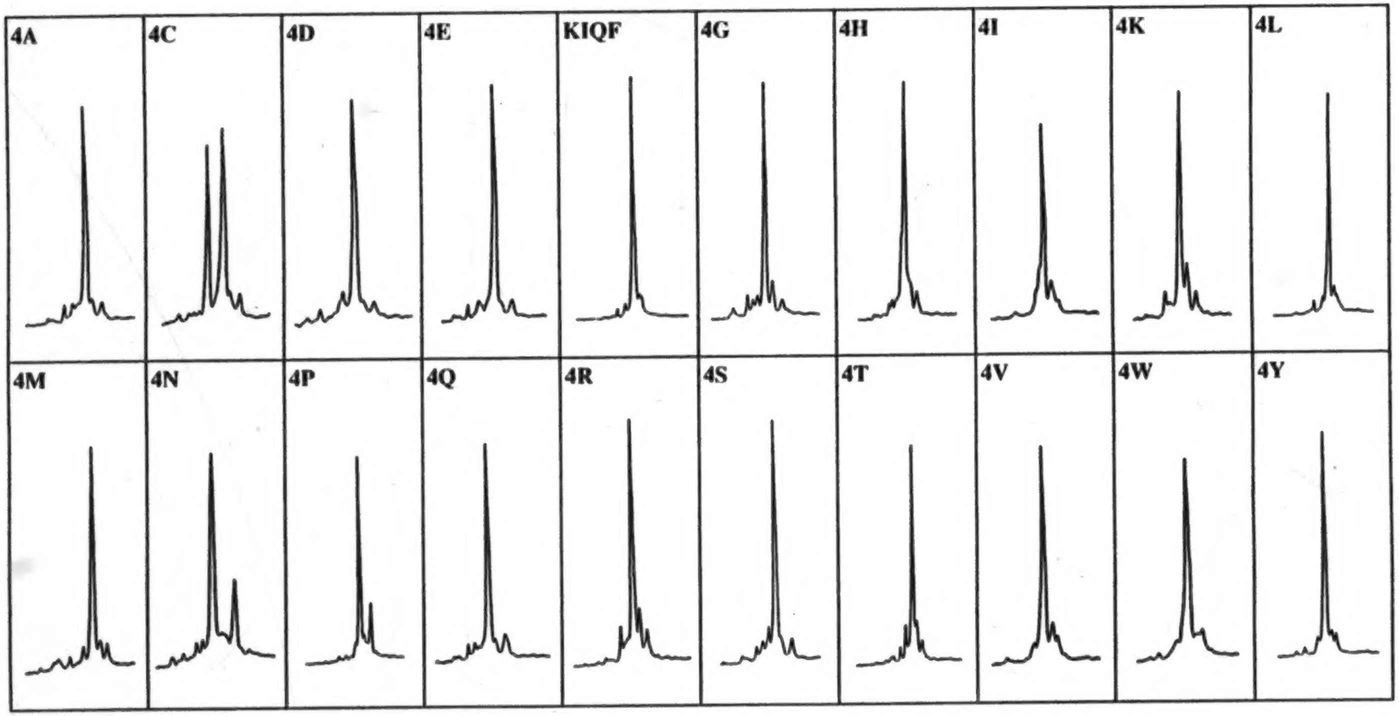
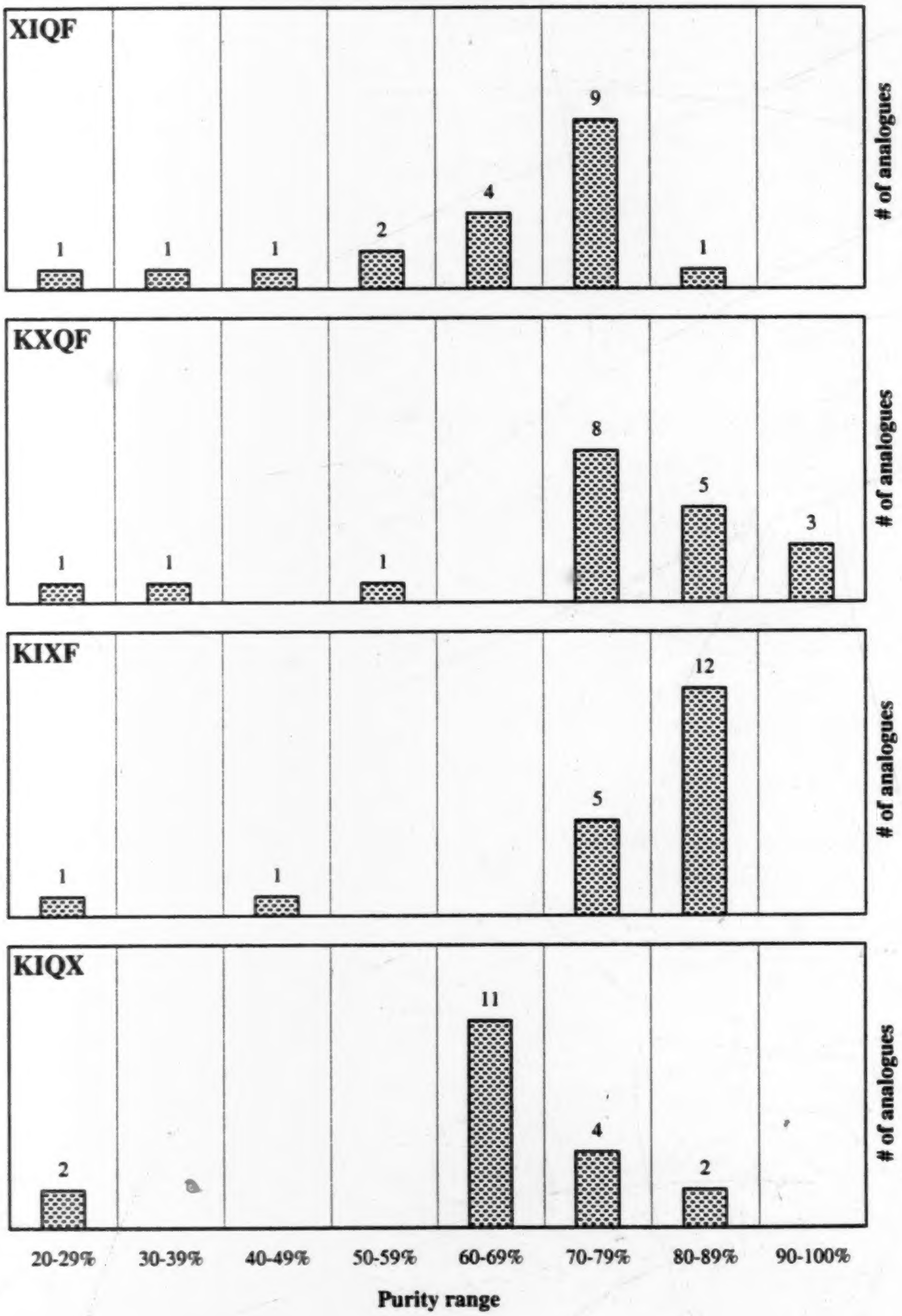


Figure 35 Quality of I-1[6-18] analogue synthesis



Chapter 5 SYNTHETIC PEPTIDE LIBRARIES (MPS APPLICATION)

5.1 Peptide Libraries and Their Uses

There is an ever increasing demand for novel compounds able to modulate a wide range of biological processes for applications in medicine and agriculture. Traditionally, these agents have been obtained by screening large collections of samples including natural products (isolated from microbiological fermentations, plant extracts, animal sources, or broths grown from soil samples) and synthetic intermediates. Peptides are attractive targets in this discovery process, despite poor transfer rates across the intestinal mucosa and cell membrane and rapid degradation and clearance, since they are key mediators of biochemical information and selectively bind and either activate or inhibit a large number of biological receptors. Screening for a desired bioactivity normally generates "leads" which are subjected to structure- or mechanism-based optimization. The "hand-crafted" development of new diagnostic or therapeutic peptides may require the synthesis and screening of hundreds or even thousands of analogues of an original active sequence; despite the development of high throughput, automated techniques to provide the necessary screening capability, the process of synthesis and testing is time-consuming (due to its essentially serial nature), costly, and has a poor success rate in commercial application: on average, only 1 in 10,000 candidates provides a marketable product.

An alternative to "rational design" has emerged with the application of combinatorial chemistry to generate an unprecedented degree of molecular diversity for selection of "ideal" entities, dramatically shortening the traditional process of discovery. **Combinatorial strategy** employs a set of chemical "building blocks" which are systematically assembled in many combinations using biological or synthetic procedures to create a large population of different molecular entities known as a **library**. The building block approach has parallels in nature, with the creation of oligonucleotides, carbohydrates, and proteins through the combination of nucleosides, sugars, and amino acids. The potential range of structural diversity accessible through assembly of even a small set of building blocks is vast: if an equal number of building blocks *b* are used in each of *x* synthetic steps in the reaction scheme, then the number of different compounds *N* generated by an ideal combinatorial synthesis is given by $N = b^x$. The basic set of 20 naturally occurring amino acids allows the construction of, for example, a tetrameric library consisting of a theoretical 20^4 (i.e. 160,000) peptides, a pentameric library with 20^5 (i.e. 3.2 million) peptides, a hexameric library containing 20^6 (i.e. 64 million) peptides, and so on. Novel assay formats relying on the inherent selectivity of biological receptor systems to retrieve the most potent ligands towards a biomolecular target are required to evaluate these libraries rapidly and identify active peptides emerging from the selection process.

Peptide libraries can be generated by biological means using recombinant technology or by chemical synthesis. In recombinant libraries, combinatorial strategy is employed to generate random synthetic oligonucleotides which are inserted into biological systems for expression; the replicative ability of the biological system is employed to amplify the DNA of active peptides and permit identification by DNA sequencing. Biological methods differ in the format of peptide presentation and the nature of the peptide-DNA linkage. Peptides may be presented as fusion products with native proteins on the surface of bacteria, as in the case of maltose receptor LamB on the outer membrane of *E. coli* (Charbit *et al.*, 1986; Brown, 1992); on the surface of bacteriophage, as in the case of minor coat protein pIII and major coat protein pVIII on filamentous phages of *E. coli* (Smith, 1985); or on plasmids, as with DNA-binding protein LacI which binds to the Lac operator on the encoding plasmid (Cull *et al.*, 1992); alternately, nascent peptides may be retrieved still linked to their encoding mRNA in polysomes using the cell-free coupled transcription/translation system of *E. coli*, and the RNA can be converted to cDNA for amplification by PCR (Mattheakis *et al.*, 1994). However, there are fundamental limitations to the biological approach: recombinant libraries are subject to inherent bias in the peptide distribution caused by the degenerate genetic code (amino acids are designated by as few as one to as many as six codons) and biological selection against certain sequences (due to structural constraints or processing inefficiency), while molecular diversity is limited to the incorporation of L-amino acids and recombinant library size is limited by the practical ability to transform DNA into the host cells or phages (unless a cell-free polysome system is employed). The format of peptide presentation may also pose problems: a peptide may be displayed in an incorrect conformation or offer reduced accessibility for screening when presented as a fusion product, the peptide may be altered significantly by exposure to different environments (cytosolic versus perisplamic compartment) with their characteristic proteolytic activities during transport to the surface, or may resist processing for transport to the surface.

Although the synthetic chemical approach to library synthesis lacks the replicative power of a biological system, it avoids inherent biological biases and dramatically expands molecular diversity by permitting the inclusion of D- and unnatural amino acids in the building block set. Combinatorial strategy is employed directly to generate random synthetic peptides by multiple peptide synthesis (MPS); multiple entities are generated simultaneously in each coupling step, resulting in an exponential increase in library size with each synthesis cycle. While mixtures of activated amino acids can be used during each coupling step to generate diversity, the resulting product distribution will be determined by the relative kinetics of the competing reactions, and since activated derivatives of sterically hindered amino acids such as Val and Ile couple at significantly slower rates, appropriate relative amino acid concentrations in the coupling mixture are employed to achieve an approximately equimolar representation in normal coupling times. The preferred mechanism for generating diversity circumvents this problem by performing separate coupling reactions with single amino acids. Syntheses can be performed in parallel by segregating the solid support into discrete fractions, each dedicated to the synthesis of a single peptide; examples include immobilized peptide arrays generated by the multipin and light-directed spatially addressable MPS methods, and free peptides generated with individual resin packets by the tea-bag MPS method. There are practical limits to the size of library which can be achieved by this procedure and the amount of record-keeping required to track individual peptides, but there is no need for sequence determination of active peptides. The alternative **split synthesis** or **divide, couple and recombine** procedure (first described by Furka *et al.*, 1991) repetitively divides the solid support into a limited number of aliquots, each dedicated to the coupling of a single amino acid, then recombines and randomizes the support by mixing before advancing to the next round of coupling (as illustrated in **Figure 36** using three amino acids and three coupling steps); this procedure allows the construction of much larger libraries, but the randomization process generates mixtures of peptides and necessitates the sequence determination of active peptides.

Library synthesis methods differ in the format of peptide presentation: a library may consist of segregated pure components or mixed pools of peptides; peptides may be immobilized (i.e. tethered) to a solid support such as plastic pins (Geysen, 1986), resin beads (Lam *et al.*, 1991), or the surface of a glass slide (Fodor *et al.*, 1991), or may be released into solution and assayed as free peptides, i.e. a **soluble peptide combinatorial library** or **SPCL** (Houghten, 1991). The format of peptide presentation ultimately determines the type of biological assay used for screening, the library size which can be practically screened, and the method for active peptide identification. Immobilized libraries were developed for immunological screening, taking advantage of the synthesis solid support to provide a ready-made immobilized component in the assay; this support can be further organized for ease of screening, as with the microtitre plate format used in the multipin method. However, immobilization requires a support suitable for both peptide synthesis and aqueous assays (i.e. characterized by low non-specific binding) and the support may interfere with screening by restricting peptide conformation (although new linkers have been designed to reduce this problem); furthermore, this presentation method has limited application outside immunology since many molecular interactions involve membrane-bound (i.e. immobilized) receptors which are unable to interact with a bound ligand. Cleavable linkers have been incorporated to allow the release of free peptides for use in solution-phase assays, but there are practical limits on the amounts of individual peptides which can be produced, especially in the case of spatially addressable libraries using the resolution provided by photolithography to permit miniaturization. Peptides synthesized on a beaded resin support using the split synthesis procedure to generate ideally **one-bead-one-sequence libraries** (reviewed in Lebl *et al.*, 1995) sacrifice the immediate identification provided by position in an array format, but individual beads carrying active peptides can be retrieved by a micromanipulator after incubation with dye- or fluorescently-labelled receptor and subjected to peptide sequencing for identification. Unfortunately, bead libraries require a positive response during screening to allow selection. **SPCLs** (reviewed in Pinilla *et al.*, 1995) provide two important advantages over immobilized libraries: versatility (since peptides can be assayed in solution, virtually any type of assay can be used) and scale (no intrinsic limitation is imposed by the format); however, the split synthesis procedure used to generate peptide diversity produces mixed pools of related structures, and a more complex iterative process (i.e. **recursive deconvolution**) is required to identify active peptides. A variety of SPCL formats have been developed by Houghten and coworkers, consisting of sets of sublibraries synthesized by the Tea bag method **(Figure 37)**. In the **positional scanning** format, single positions (represented by the notation **O**) are defined throughout a sequence of fixed length and all remaining positions are composed of mixtures of amino acids (represented by **X**), so that the number of sublibraries is equal to the number of residues in the sequence; this type of SPCL provides information on the most active amino acid in each position, the replaceability of each position and its relative importance. In the **dual position** format, the first two positions are defined and the remaining positions are composed of mixtures of amino acids; once the library of mixtures is screened in an assay, an iterative process is used to define the remaining mixture positions sequentially, each stage of screening involving resynthesis and evaluation of 20 sublibraries containing a reduced number of peptide sequences. One advantage of the iterative process is the enrichment resulting from the declining peptide diversity at each stage.

Different library formats lend themselves to different applications. Array formats generated by parallel synthesis are particularly suited for analogue libraries designed on the basis of some structural knowledge of peptide ligands or their target receptors. Multipin synthesis is commonly employed for the preparation of analogue libraries for **epitope analysis**: continuous epitopes can be identified using the complete set of overlapping peptides derived from an antigen's sequence to determine epitope location and its boundaries, while a comprehensive set of epitope analogues can be used to identify residues critical for binding and intolerant of substitution (i.e. **replacement set analysis**), and retrieve sequences with higher affinities than the starting sequence. Analogue libraries are used in a similar fashion for structure-function studies. In contrast, "random" libraries are designed for novel ligand discovery programs and employ screening strategies requiring no previous knowledge of active peptide structural requirements (for example, the search for **mimotopes** which are mimetics of discontinuous, conformation-dependent epitopes). The goal is to create the greatest molecular diversity in the library, but there are practical limits to the vast number of combinatorial possibilities which can be synthesized and so care must be exercised in the choice of template for randomizing; design parameters include the choice of peptide length (since diversity increases with length) and possible incorporation of fixed "scaffolds" of constraining residues to increase affinity (although this reduces conformational diversity). SPCL versatility may prove most useful, as soluble peptides produced in sufficient quantity may be screened in a wide variety of binding or functional assays.

Figure 36 **Schematic diagram of split synthesis procedure**

(adapted from Lam *et al.*, 1991)

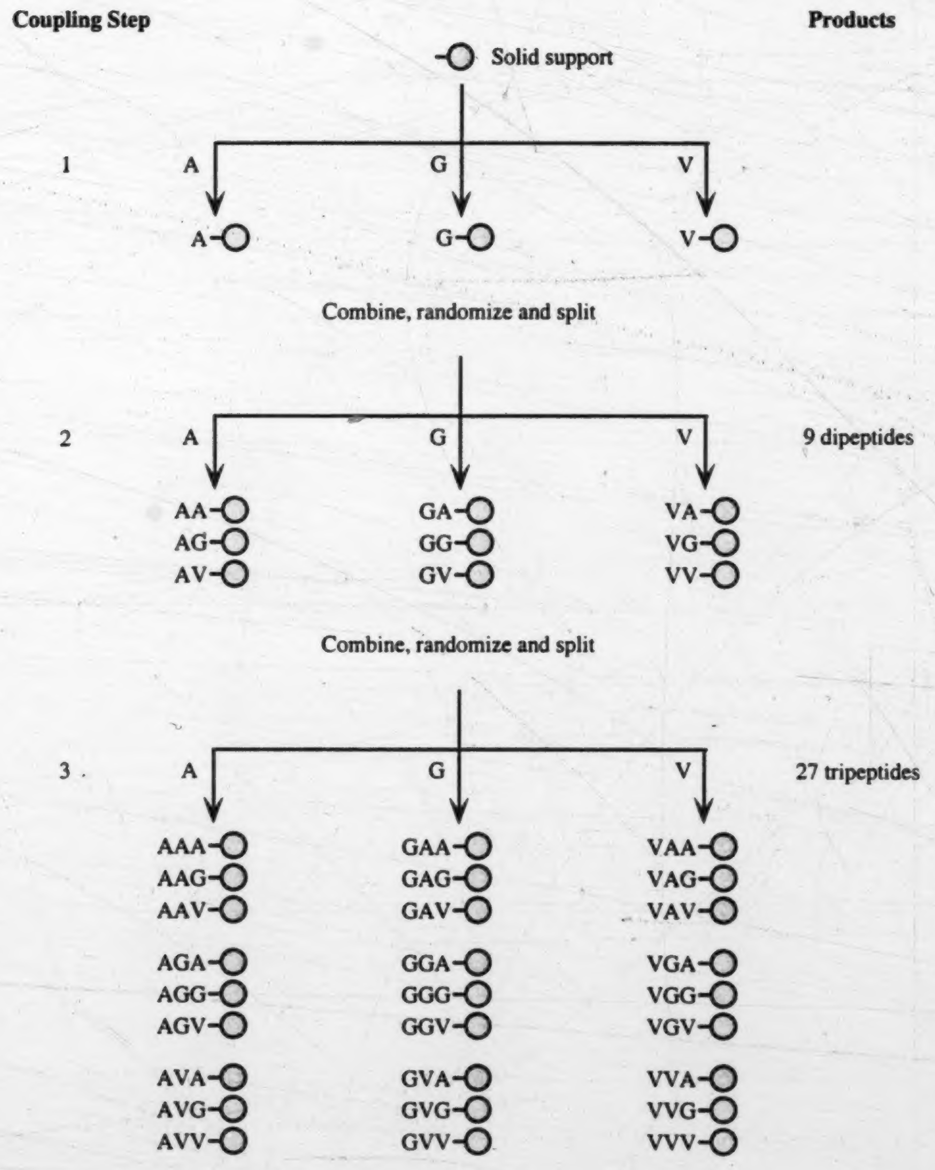

Coupling Step
Products
Solid support
1
A
G
V
A
G
V
Combine, randomize and split
2
A
G
V
9 dipeptides
AA
AG
AV
GA
GG
GV
VA
VG
VV
Combine, randomize and split
3
A
G
V
27 tripeptides
AAA
AAG
AAV
AGA
AGG
AGV
AVA
AVG
AVV
GAA
GAG
GAV
GGA
GGG
GGV
GVA
GVG
GVV
VAA
VAG
VAV
VGA
VGG
VGV
VVA
VVG
VVV

Figure 37 Common SPCL Formats

Library	No. of peptide mixtures	Theoretical no. of individual peptides per mixture	Theoretical total no. of peptides
Positional scanning			
O_1 X X X X X	20	3.2×10^6	6.4×10^7
X O_2 X X X X	20	3.2×10^6	6.4×10^7
X X O_3 X X X	20	3.2×10^6	6.4×10^7
X X X O_4 X X	20	3.2×10^6	6.4×10^7
X X X X O_5 X	20	3.2×10^6	6.4×10^7
X X X X X O_6	20	3.2×10^6	6.4×10^7
Dual defined			
O_1 O_2 X X X X	400	1.6×10^5	6.4×10^6

O = position defined with a single amino acid; **X** = position composed of a mixture of amino acids. Amino acid set consists of all 20 natural amino acids.

5.2 Construction of a Representative Peptide Library

A novel approach to reducing library size while maintaining diversity involves replacing the 20 naturally occurring amino acids with 10 representative amino acids displaying the basic physico-chemical properties associated with the larger set of sidechains **(Table 11)**. For example, Glu and Lys can be chosen to represent acidic and basic sidechains, respectively, Gln to represent uncharged amide derivatives, Phe to represent aromatic sidechains and Ser to represent hydroxyl aliphatic sidechains, while conformational aspects can be represented by Gly (whose sidechain has no steric hindrance), Pro (having restricted conformation as an imino acid), and Ile (possessing a β-branched sidechain). Potential synthesis problems such as difficult couplings or sidechain modifications of certain amino acids (such as Cys and Trp), which may affect adversely the composition of the library, can also be avoided by eliminating these residues. The reduction in library size is significant: as demonstrated in **Table 12**, the representative approach provides a 64-fold reduction in peptide numbers in a hexapeptide library, and an even greater advantage in relative population size is realized as peptide length increases. A **representative library** also confers a concentration advantage since the library contains fewer members for a given peptide length, permitting the use of lower affinity antibodies or receptors in the screening process.

To investigate the feasibility of this approach, a soluble peptide combinatorial library (SPCL) format was chosen to permit use of a wide range of screening assays and allow concentration adjustments to optimize assay performance. Peptide length was based on the 6 to 10 residue length common for epitope identification (although there is a growing recognition of the importance of shorter 4 to 6 residue sequences in protein-protein interactions and protein targeting, as seen with the PP-1 binding motif); selection of a hexameric peptide represents a compromise between maximizing diversity (increasing with each additional residue) and minimizing synthesis and screening requirements (since each additional residue represents a 10-fold increase in library population). Two types of **representative combinatorial peptide libraries (RPCLs)** with differing techniques for identifying active peptides were constructed: a set of **dual-defined RPCLs**, which predict active hexapeptides from combinations of active dipeptide sequences, and a **triple-defined RCPL**, which relies on an iterative strategy for peptide identification.

Initially, a set of 3 complementary dual-defined RPCLs denoted by $Ac\text{-}O_1O_2X_3X_4X_5X_6\text{-}NH_2$, $Ac\text{-}X_1X_2O_3O_4X_5X_6\text{-}NH_2$, and $Ac\text{-}X_1X_2X_3X_4O_5O_6\text{-}NH_2$ were synthesized at 6 μmol scale on the original Holm and Meldal synthesis unit (a reaction block containing a 96-well array in microtitre plate format) using representative Fmoc-amino acid Opfp esters at 10-fold excess for defined O_nO_{n+1} positions and double couplings of mixtures of 0.1 equivalents of each ester for X_n positions (Wong *et al.*, 1994). Initial synthesis requirements were substantial, as each library consisted of 100 (10^2) peptide mixtures containing 10,000 (10^4) different peptides with acetylated N-termini and amidated C-termini; however, this type of library avoids the effort involved in an iterative strategy for peptide identification by combining the active dipeptide results from each library in the set to predict active hexapeptides for re-synthesis and screening. Wong's experience with the dual-defined representative library prompted the construction of a novel triple-defined representative library denoted by $Ac\text{-}O_1O_2O_3X_4X_5X_6\text{-}NH_2$, consisting of 1000 ($10^3$) peptide mixtures composed of 1000 (10^3) hexapeptides with acetylated N-termini and amidated C-termini per mixture. The introduction of a third defined position was designed to provide potentially greater specificity and selectivity, while the substitution of an iterative procedure of active peptide identification for the predictive approach of the dual-defined libraries was intended to ensure identification of all (complete) active sequences; the third defined position also eliminated one round of the iterative procedure. The triple-defined library was generated by the split-synthesis approach **(Figure 38)**, using tedious manual synthesis to achieve a 50 mmol scale of synthesis (considerably greater than possible on the Holm and Meldal instrument) for the addition of the first 5 residues; the library was rapidly completed on the 100-reactor module of the newly developed multiple peptide synthesizer prototype in 10 rounds of synthesis, each round corresponding to the splitting and coupling of 10 of the 100 O_2O_3XXX resin-bound mixtures (and each reactor representing one $O_1O_2O_3$XXX mixture at 50 μmol scale). In order to reduce costs at so large a scale of synthesis, batch-mode MBHA resin was selected in place of continuous flow NovaSyn PR resin, and a pre-formed activated Rink amide handle was attached. The standard protocol for MPS synthesis was followed throughout: deprotection by 20% piperidine in DMF; Fmoc-amino acid sidechain protecting groups of OtBu (Asp, Glu), tBu (Ser, Thr,Tyr), Boc (Lys), Trt (Cys and His), and Pmc (Arg); *in situ* activation of Fmoc-amino acids to generate OBt esters from HBTU; N-terminal acetylation using excess acetic anhydride; cleavage by modified Reagent K; and cold ether precipitation of peptides. However, 10-fold excess Fmoc-amino acids and extended 90-120 minute coupling times (rather than nitrogen gas sparging) were employed to ensure high quality synthesis on the batch resin, and the resulting crude peptide mixtures were neutralized by lyophilization from 10 mM NH_4HCO_3 before use in pH-sensitive ELISA assays. Each $O_1O_2O_3$XXX mixture was redissolved in 2.5 mL of water to give a theoretical total peptide concentration of 20 mM and an individual peptide concentration of 20 μM before storage at -80°C.

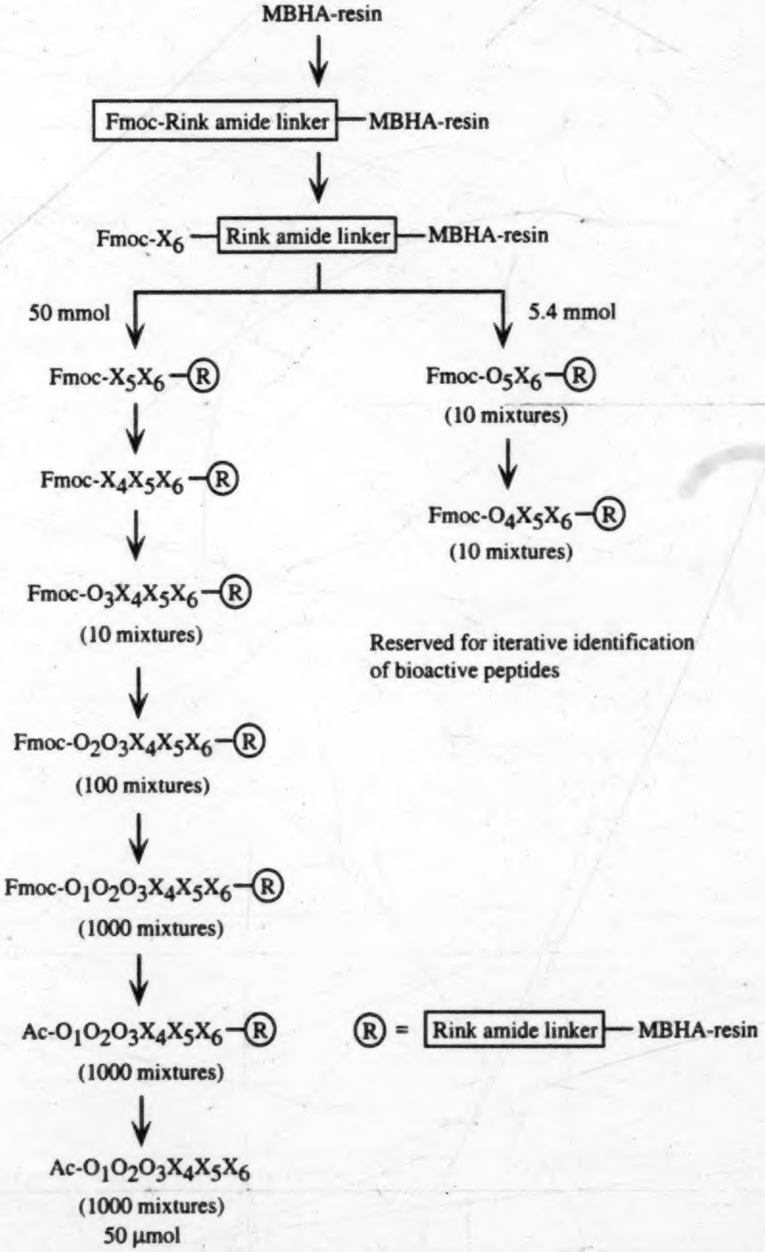
Table 11 Selected Representative Amino Acids

Representative amino acid	Physico-chemical properties of sidechains	Amino acids represented
Ala	small hydrophobic	
Glu	acidic	Asp
Phe	aromatic	Trp, Tyr
Gly	no steric hindrance	
Ile	β-branched larger hydrophobic	Val Met
Lys	basic	His, Arg
Leu	larger hydrophobic	Met
Pro	restricted conformation	
Gln	amide	Asn
Ser	hydroxyl	Thr

Table 12 Reduction in Library Size by the Representative Approach

Peptide length	Number of peptides in library	
	Complete library	Representative library
2	$20^2 = 400$	$10^2 = 100$
3	$20^3 = 8{,}000$	$10^3 = 1{,}000$
4	$20^4 = 160{,}000$	$10^4 = 10{,}000$
5	$20^5 = 3.2$ million	$10^5 = 100{,}000$
6	$20^6 = 64$ million	$10^6 = 1$ million
7	$20^7 = 1.28$ billion	$10^7 = 10$ million
8	$20^8 = 25.6$ billion	$10^8 = 100$ million

Figure 38 Overview of Triple-Defined Representative Library Synthesis


MBHA-resin
Fmoc-Rink amide linker — MBHA-resin
Fmoc-X_6 — Rink amide linker — MBHA-resin
50 mmol
5.4 mmol
Fmoc-X_5X_6—Ⓡ
Fmoc-$X_4X_5X_6$—Ⓡ
Fmoc-$O_3X_4X_5X_6$—Ⓡ
(10 mixtures)
Fmoc-$O_2O_3X_4X_5X_6$—Ⓡ
(100 mixtures)
Fmoc-$O_1O_2O_3X_4X_5X_6$—Ⓡ
(1000 mixtures)
Ac-$O_1O_2O_3X_4X_5X_6$—Ⓡ
(1000 mixtures)
Ac-$O_1O_2O_3X_4X_5X_6$
(1000 mixtures)
50 µmol
Fmoc-O_5X_6—Ⓡ
(10 mixtures)
Fmoc-$O_4X_5X_6$—Ⓡ
(10 mixtures)
Reserved for iterative identification of bioactive peptides
Ⓡ = Rink amide linker — MBHA-resin

5.3 Representational Library Screening and Assessment

The feasibility of the representative approach was tested with the set of dual-defined RPCLs in an epitope mapping of murine monoclonal antibody PK99H, an IgG which recognizes a 7-residue epitope DEQFIPK located in sequence 134-140 of the C-terminal region of *Pseudomonas aeruginosa* strain K (PAK) pilin (Wong *et al.*, 1992). *P. aeruginosa* is a significant opportunistic pathogen in individuals with a disrupted natural host-defence mechanism, such as burn victims and cancer patients (Bodley *et al.*, 1983), and pilus-mediated bacterial adherence to epithelial cell surfaces is one of three known adherence mechanisms leading to pathogenesis (Doig *et al.*, 1988). The dual-defined libraries were screened by competitive ELISA to determine their ability to inhibit the interaction of PK99H with a 17-residue intrachain disulfide-bridged (oxidized) PAK pilin peptide containing the 7-residue epitope; this N^{α}-acetylated peptide has the sequence of Ac-KCTSDQDEQFIPKGCSK-OH corresponding to the C-terminal region of PAK pilin functioning as the adherence binding domain (residues 128-144). 96-well microtitre plates were coated with the 17-residue PAK pilin peptide (200 μM, 80 μL per well) in 10 mM sodium carbonate buffer (pH 9.5), incubated 6 hours at room temperature, and blocked against non-specific adsorption of antibody with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) overnight at 4°C. Library solutions (diluted to a final concentration of 8 mM) were pre-incubated with PK99H (5 ng/mL final concentration) in 50 mM PBS containing 0.05% BSA (pH 7.4) at room temperature for 1 hour; solution mixtures (80 μL) were transferred to the corresponding wells and incubated at 37°C for 2 hours. Bound antibody was detected by goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Laboratories, California) using 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) as a substrate. The secondary antibody-enzyme conjugate (100 μL at 5000-fold dilution) was added to the wells and incubated at 37°C for 2 hours, after which freshly prepared substrate solution (100 μL) consisting of 10 mM sodium citrate (pH 4.2) with 0.03% v/v hydrogen peroxide and 1 mM of the substrate was added and incubated at room temperature for 30 minutes. The absorbance of each well was measured at 405 nm by a Titertek Multiskan Plus MKII microplate reader (FlowLab Inc., McLean, Virginia) and compared with a "blank" well (i.e. lacking competitor peptide) to determine the % inhibition provided by the library solution.

Using a cut-off at 50% inhibition to select significant responses and locate 2-residue sequences important for antibody binding (since a 100% cut-off is wasteful of the library), 9 "hits" (EQ, QQ, QA, AF, GQ, SL, EF, GL and QL) were obtained in the O_1O_2XXXX library, 1 hit (FI) in the XXO_3O_4XX library, and 5 hits (PK, IG, PL, LG and IL) in the $XXXXO_5O_6$ library **(Figure 39)**. The best hits in these libraries were EQ, FI, and PK respectively, which, when combined in order, constitute the native PAK epitope sequence EQFIPK. In addition to their success in identifying the known epitope sequence, the RPCLs were also able to represent a range of other sequences: substitution of represented amino acids for representative residues in EQFIPK produced 4 peptides (DQFIPK, EQWIPK, EQFMPK and EQFVPK) having I_{50} values within an order of magnitude of the native sequence, including 2 peptides (EQWIPK and EQFMPK) with a slightly higher affinity for PK99H than the native sequence **(Table 13)**. The results with EQFIPK and two additional combinations QQFIPK and QAFIPK not found in the native PAK sequence demonstrate that positions 1,3, and 4 permit the use of the representative approach without substantially altering binding affinity to PK99H: Glu and Gln were able to represent Asp and Asn, F was able to represent Trp, and I could represent Met and Val. Limitations to the representative approach do exist: Arg and His could not be substituted by their representative amino acid Lys at the critical position 6 (since Lys is charged at the assay pH of 7.4 while His is not, and Arg has a more bulky sidechain than Lys), and problems arise where residue sidechain contributions to antibody-antigen interactions do not depend only on their physico-chemical properties, as demonstrated in the second position of the epitope where substitution of Gln (considered nonessential for binding by alanine replacement analysis) with amino acids other than Ala or Cys strongly affected peptide binding to PK99H. Another limitation is caused by the combination of active dipeptides to predict complete active sequences; the large difference in I_{50} value of the peptide AFFIPK (1100 μM) compared with the native sequence (1.3 μM) suggests that the first two residues mapped (AF) were not in combination with the last four residues (FIPK) to give the initial positive hit, and combinations with the next best hits for positions 5 and 6 (AFFIIG and AFFIPL) were not sufficiently soluble to test in the assay.

The success of the representative approach and the limitations of dual-defined library prediction of active sequences led to the comparative screening of the novel triple-defined RPCL by the same competitive ELISA with monoclonal antibody PK99H and oxidized PAK pilin (128-144), using a library dilution an order of magnitude smaller to account for the differing individual peptide concentration (as each mixture in the triple-defined RPCL contains only 1000 diferent peptides, compared to the 10,000 peptides per mixture in the dual-defined RPCLs). With this type of library, identification of active peptides proceeds through an iterative strategy: at each iterative step, peptide mixtures having one more position defined are synthesized and screened. The first iterative step with the triple-defined library detects active peptides with the frst 3 positions defined. Using a cut-off at 50% inhibition to select significant responses, the library mixture EQFXXX containing the native PAK epitope sequence EQFIPK was correctly identified **(Figure 40)**. Indeed, this was the sole hit, although 4 other library mixtures (SLGXXX, QQFXXX, QFQXXX and EIKXXX, in order of inhibitory effect) provided only slightly less competition, and one of these (QQFXXX) can be considered the equivalent of the QQ and FI combination obtained in the dual-defined RPCLs O_1O_2XXX and XXO_3O_4XXX. The selective advantage of the triple-defined RPCL can be seen in the low "background" of the other responses, allowing hits to be distinguished easily; the only exception appears to be the series in which O_3 was defined as Ser, and potential hits in this series were later identified as false positives when screened on a common microtitre plate with EQFXXX. Due to very limited supplies of monoclonal antibody PK99H, screening could not be continued beyond this point.

Monoclonal antibody PK99H is a strain-specific antibody, recognizing only strain K (PAK) pilin. The existence of several strains of *P. aeruginosa* complicates the development of an effective vaccine, necessitating the preparation of a "cocktail" vaccine or the development of a concensus sequence for complete protection. Sequences of the C-terminal regions (i.e. adherence domains) from residues 128 to 144 of several *P. aeruginosa* strains are presented in **Figure 41**, along with the corresponding sequence from exoenzyme S (EXOS), showing the degree of shared homology; the latter is of interest since *P. aeruginosa* adherence to epithelial cells can also proceed by the mechanism of exoenzyme S - mediated binding. For this reason, the triple defined RPCL was screened by competitive ELISA using cross reactive monoclonal antibody EXOS#50 (another IgG) in place of the strain-specific PK99H. This posed a considerable challenge to the representative library, requiring the successful representation of 9 amino acids (D,H,M,N,R,T,V,W, and Y, omitting C which has no representative) for the detection of lower affinity cross reactive binding sequences, in addition to C, T, and N within the homologous region of the EXOS sequence. The previously described competitive ELISA protocol was employed, substituting PAK pili (0.5 μg per well) for the 17-residue PAK pilin and using a final antibody concentration of 3.5 µg/mL (reflecting its lower affinity for the pili) and initial library mixture dilutions of only 5-fold. As anticipated, multiple hits were obtained in the first round of screening, and the selective advantage was less apparent (**Figure 42**); once again, the series with O_3 defined as Ser generated a disproportionately high background. The sheer number of hits emphasized the importance of limiting selection carried into the next round of an iterative strategy of peptide identification. As an alternative to retesting positive hits (and expending library supplies in a repetitive manner), a simple "filter" was devised based on the positional frequency at which representative amino acids occurred in each of the 3 defined positions of hits (**Table 14**). Each hit was assigned a **multiple positional frequency**, a quasi "probability factor" calculated as the product of its O_1, O_2 and O_3 residue frequencies; for example, the product frequency of hit IIAXXX is 0.29 x 0.33 x 0.42 = 0.0402, i.e. the largest frequency (but not the corresponding highest % inhibition). Although this filter is a very rough statistical tool, it was able to differentiate 15 hits (**Figure 43**) from which were selected the top ten $O_1O_2O_3$ sequences in terms of inhibitory effect: LEA, QIA, LGA, LQA, SIA, LAA, QSQ, QQA, QIQ and IQS; none of these selections correspond to real or represented sequences found in the corresponding region of EXOS. Indeed, only one tripeptide sequence found in the homologous region (SSA) qualified as a hit, rating poorly in comparison with the best hits. The filter-based selection could be distinguished from a selection based solely on % inhibition, since 3 additional sequences with higher inhibitory effects but proportionally lower multiple positional frequencies were rejected by the filter for further deconvolution.

The effectiveness of this selective technique was examined in the next round of the iterative strategy. Quadruple-defined "sublibraries" (denoted by $O_1O_2O_3O_4XX$) were synthesized from $O_4X_5X_6$-resin stock reserved during synthesis of the triple-defined RPCL, and were screened at a 10-fold higher dilution to take into account the concentration advantage occurring as mixture diversity declined from 1000 (10^3) to 100 (10^2) different peptides per mixture (results presented in **Figure 44**). Only seven of the original ten $O_1O_2O_3XXX$ hits generated successive $O_1O_2O_3O_4XX$ hits (the remainder were classified as false positives); in addition, one of the original hits (QIQXXX) generated multiple hits upon addition of a fourth defined residue, suggesting that this position may not be critical to binding and may tolerate a wide range of substitution (including Ala, Ile, Leu, Gln and Ser). Carrying on to the next round, quintuple-defined sublibraries ($O_1O_2O_3O_4O_5X$) were synthesized from O_5X_6-resin stock reserved during the original library synthesis and screened at a 100-fold higher dilution than in the original round of screening. As demonstrated in **Figure 45**, only one of the previous $O_1O_2O_3O_4XX$ hits (QIQSXX) generated a successive $O_1O_2O_3O_4O_5X$ hit (QIQSIX), although 3 mixtures derived from the QIAQXX hit approached the cut-off and 1 mixture derived from the SIAAXX hit provided a disproportionate inhibitory response relative to the 9 other residues at the O_5 position. The apparent loss of so many potential hits suggests the representative nature of the library may pose a serious problem in the latter stages of an iterative strategy for active peptide identification - the increasing restriction in library diversity brought about by the use of representative amino acids may prevent successful deconvolution.

This hypothesis was tested by resynthesizing the $QIQO_4XX$ set of sublibraries using an expanded set of 19 amino acids for residue addition at positions 4, 5 and 6 (excluding only Cys for ease of synthesis); double couplings of mixtures of 0.1 equivalents of each amino acid were employed at undefined (X) positions 5 and 6 to achieve an approximately equimolar distribution. The screening results presented in **Figure 46** show unexpectedly high responses, suggesting the screening was performed with insufficiently diluted library solutions, but the overall profile allows a preliminary assessment of the representative approach: screening of the "complete" library selected QIQTXX as the highest ranking hit, in agreement with the selection of the representative equivalent QIQSXX, confirming the ability of Ser to represent Thr at this position. Declining inhibition was observed with the addition of Tyr, Trp, Leu, Ser, Gln, Asn, Val, Phe and Met in that order. Gln appears to have functioned as a satisfactory representative of Asn, according to the similar inhibitory effects, but Phe under-represented the inhibitory effects of Tyr and Trp at this position and Ile could not act as a suitable representative of Met and Val. Selecting the single best hit for investigation, the QIQTO$_5$X set of sublibraries was prepared using all 19 amino acids, and the screening results defining the fifth position clearly demonstrate the problem of restricted diversity (**Figure 47**). The 5 top-ranking hits (QIQTDX, QIQTGX, QIQTEX, QIQTQX and QIQTYX) do not include the sole apparent hit (QIQSIX) detected by the representative library.

The screening results obtained with the cross-reactive monoclonal antibody EXOS#50 illustrate the proper role for the representative library: an initial rapid screening tool, designed to compress a very large range of potential active sequences in a single screening step while effectively maintaining diversity through the use of suitable representative amino acids. This initial screening must then be followed by a conventional iterative strategy for active peptide identification, in which deconvolution is performed one position at a time using the full range of amino acids to avoid restricting diversity as binding affinity increases with each successive addition and it becomes increasingly difficult to represent a particular residue. Fortunately, the disadvantage posed by the increased synthesis requirements can be offset in part by the use of the multiple peptide synthesizer for rapid resynthesis of sublibraries.

Figure 39 Screening of Dual-Defined RPCLs :

Competitive inhibition of PK99H binding to PAK[128-144]

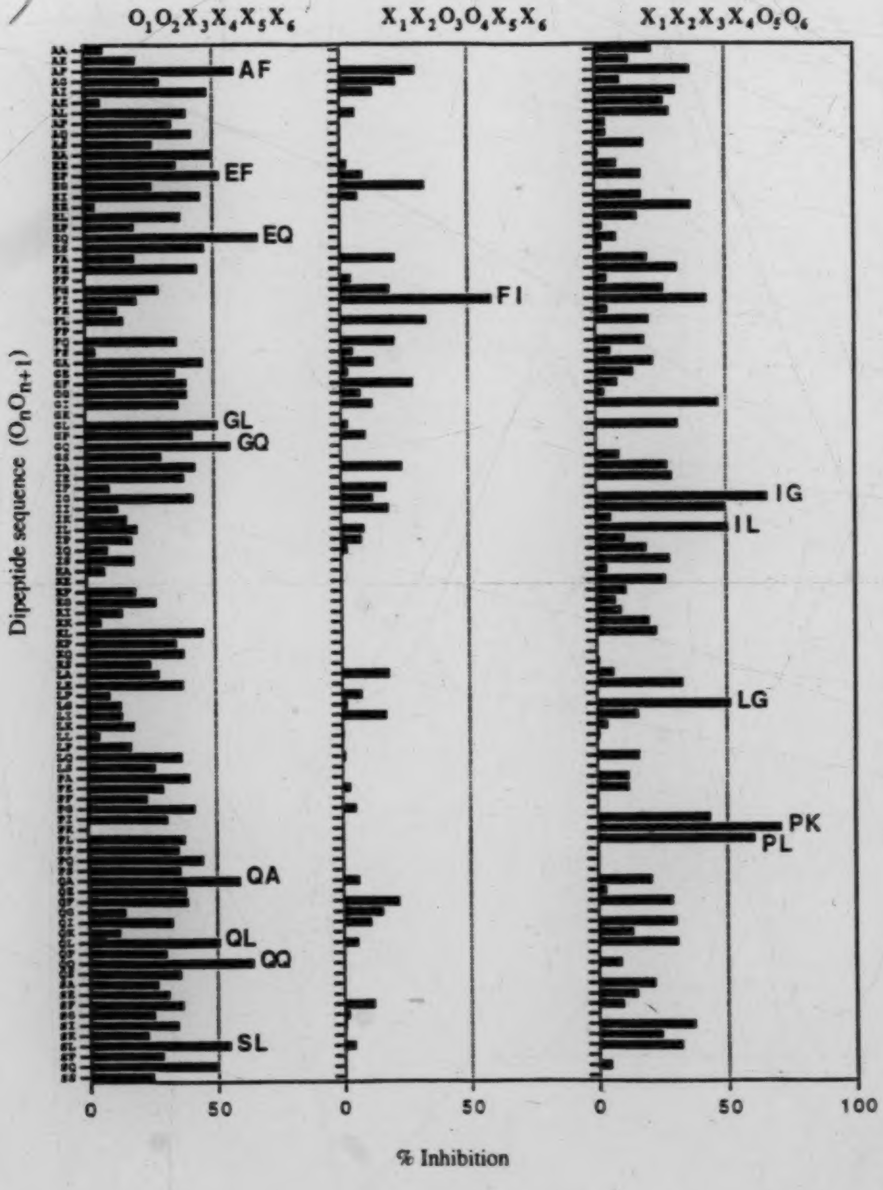


Table 13 **Substitution of Corresponding Represented Amino Acids in Active Sequences**

Sequence	I_{50}(mM)
$\boxed{\text{E Q F I P K}}$	1.3
$\boxed{\text{D}}$ Q F I P K	2.3
E $\boxed{\text{N}}$ F I P K	190
E Q $\boxed{\text{Y}}$ I P K	190
E Q $\boxed{\text{W}}$ I P K	0.9
E Q F $\boxed{\text{M}}$ P K	1.1
E Q F $\boxed{\text{V}}$ P K	3.4
E Q F I P $\boxed{\text{H}}$	620
E Q F I P $\boxed{\text{R}}$	>730
$\boxed{\text{Q Q F I P K}}$	1.3
$\boxed{\text{N}}$ Q F I P K	12.0
$\boxed{\text{Q A F I P K}}$	2.0
$\boxed{\text{N}}$ Q F I P K	14.0

Sequences of original active peptides are boxed, as are substitutions of represented amino acids.

Peptides are N^{α}-acetylated and C-amidated.

I_{50} values are peptide concentrations required for 50% inhibition of PK99H binding to PAK[128-144].

Figure 40 Screening of $O_1O_2O_3X_4X_5X_6$ RPCL :

Competitive inhibition of PK99H binding to PAK[128-144]

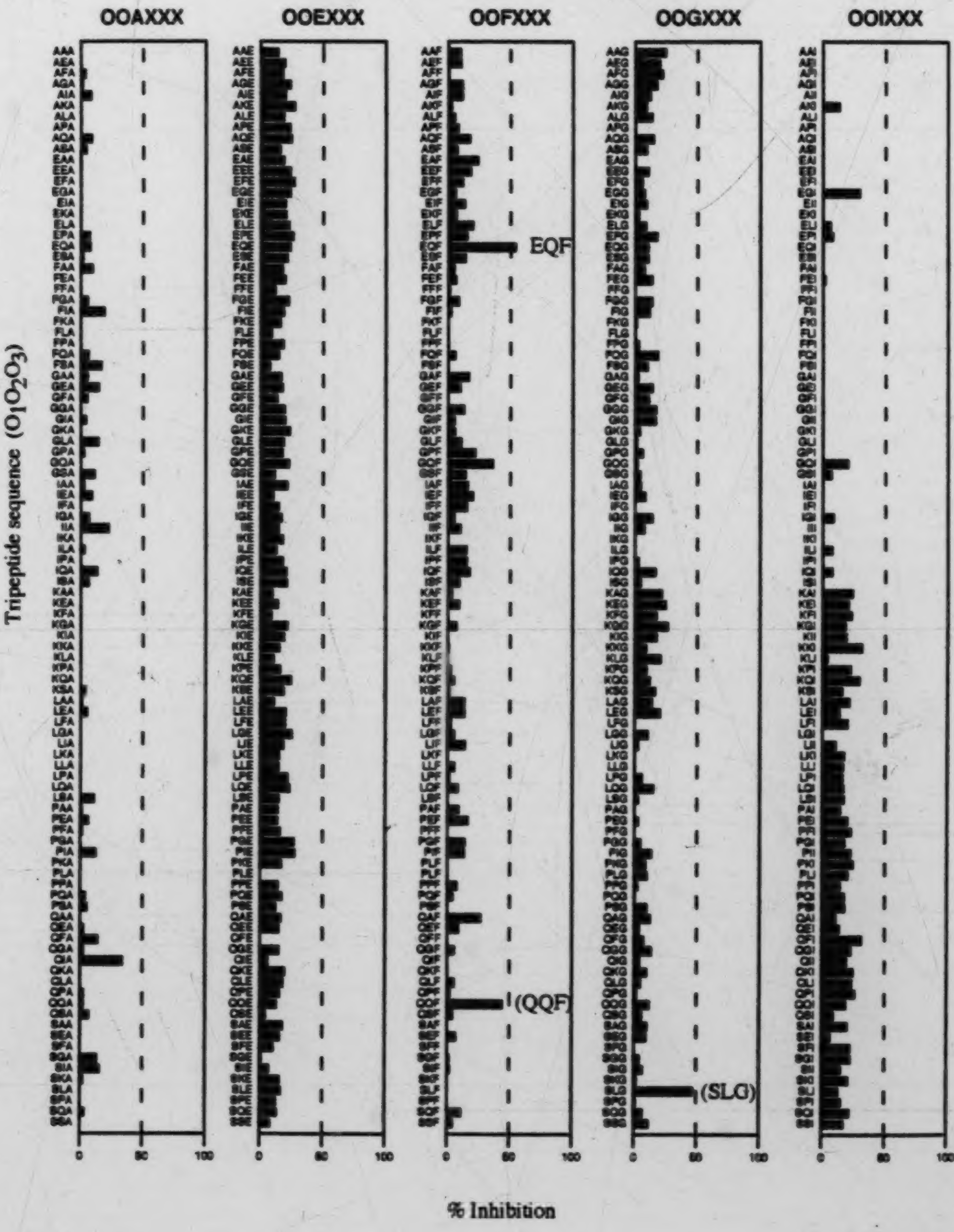


Figure 40 **Screening of $O_1O_2O_3X_4X_5X_6$ RPCL (continued) :**

Competitive inhibition of PK99H binding to PAK[128-144]

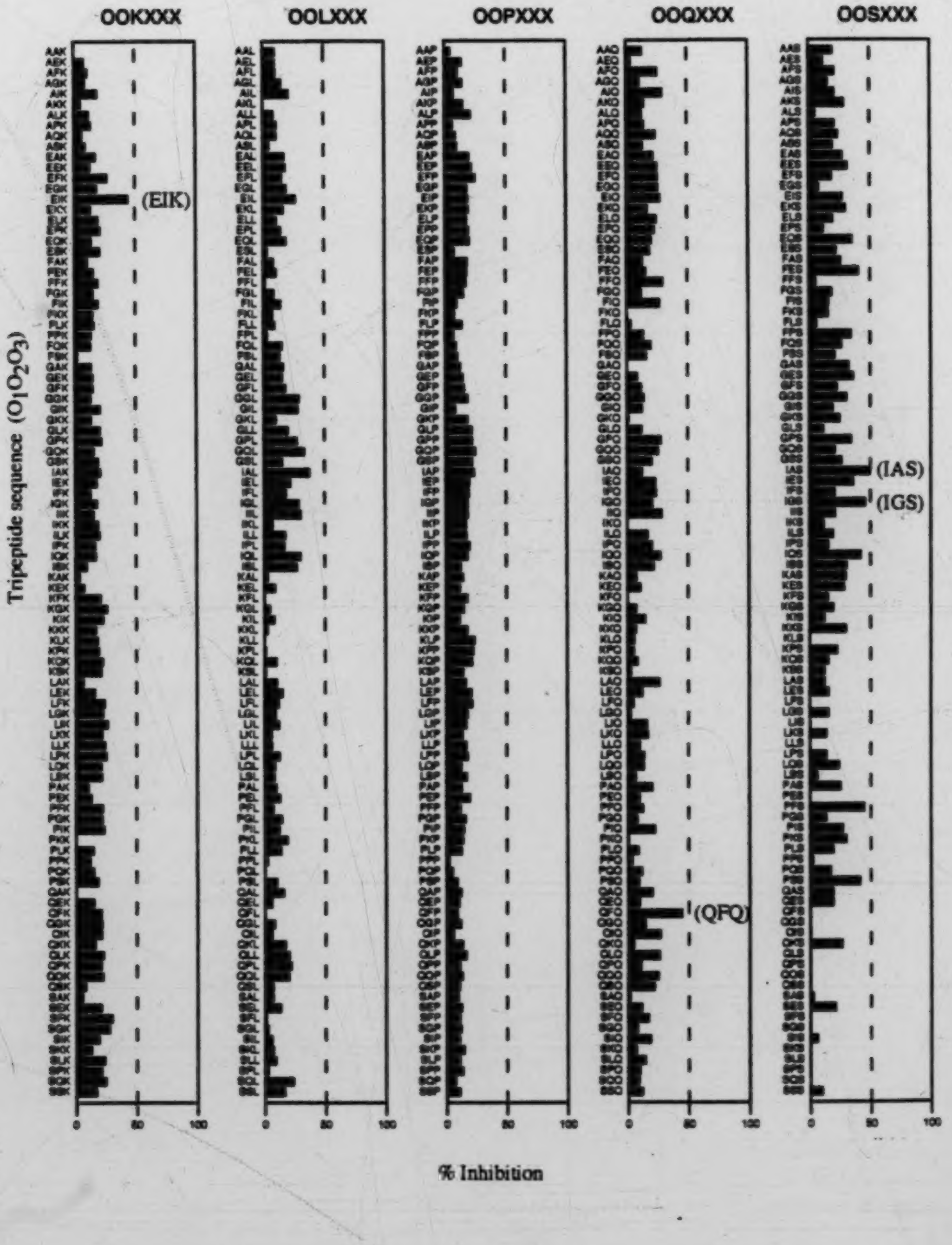


Figure 41 Comparison of Exoenzyme S and the C-Terminal Region Sequences of Eight *Pseudomonas aeruginosa* Pilin Strains

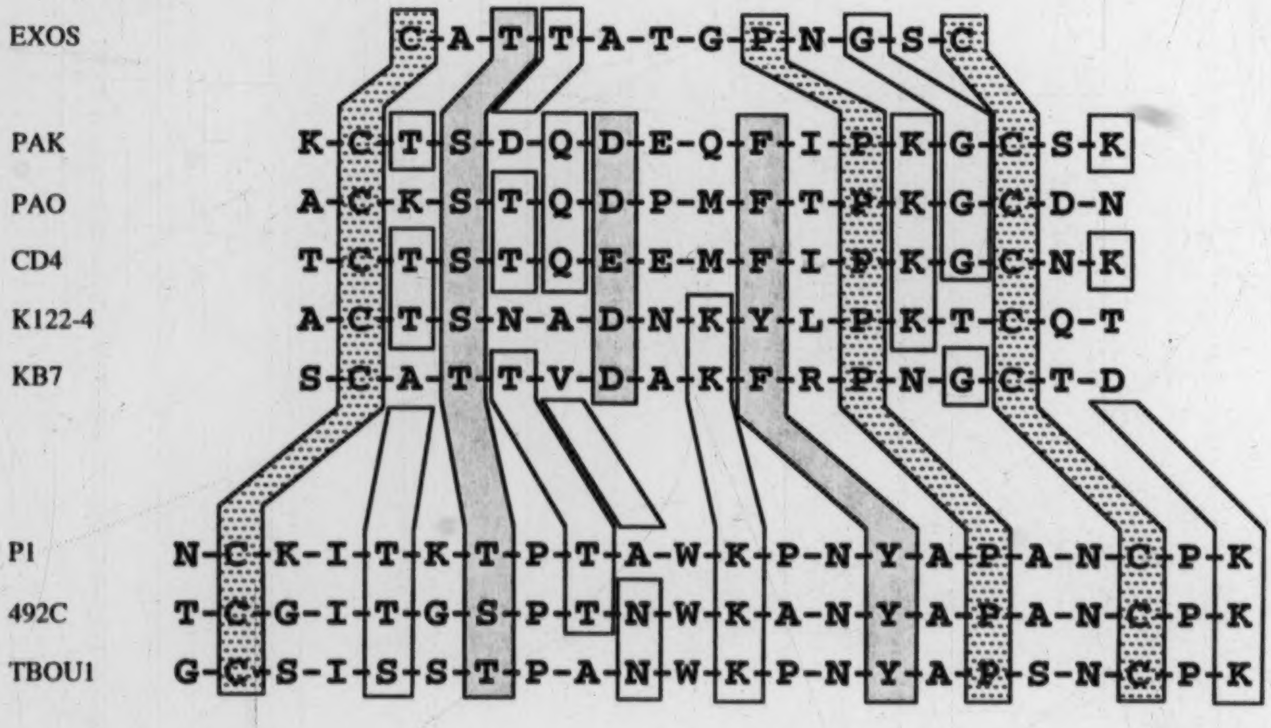

EXOS C-A-T-T-A-T-G-P-N-G-S-C
PAK K-C-T-S-D-Q-D-E-Q-F-I-P-K-G-C-S-K
PAO A-C-K-S-T-Q-D-P-M-F-T-P-K-G-C-D-N
CD4 T-C-T-S-T-Q-E-E-M-F-I-P-K-G-C-N-K
K122-4 A-C-T-S-N-A-D-N-K-Y-L-P-K-T-C-Q-T
KB7 S-C-A-T-T-V-D-A-K-F-R-P-N-G-C-T-D
P1 N-C-K-I-T-K-T-P-T-A-W-K-P-N-Y-A-P-A-N-C-P-K
492C T-C-G-I-T-G-S-P-T-N-W-K-A-N-Y-A-P-A-N-C-P-K
TBOU1 G-C-S-I-S-S-T-P-A-N-W-K-P-N-Y-A-P-S-N-C-P-K

Figure 42 Screening of $O_1O_2O_3X_4X_5X_6$ RPCL :

Competitive inhibition of EXOS#50 binding to PAK pili

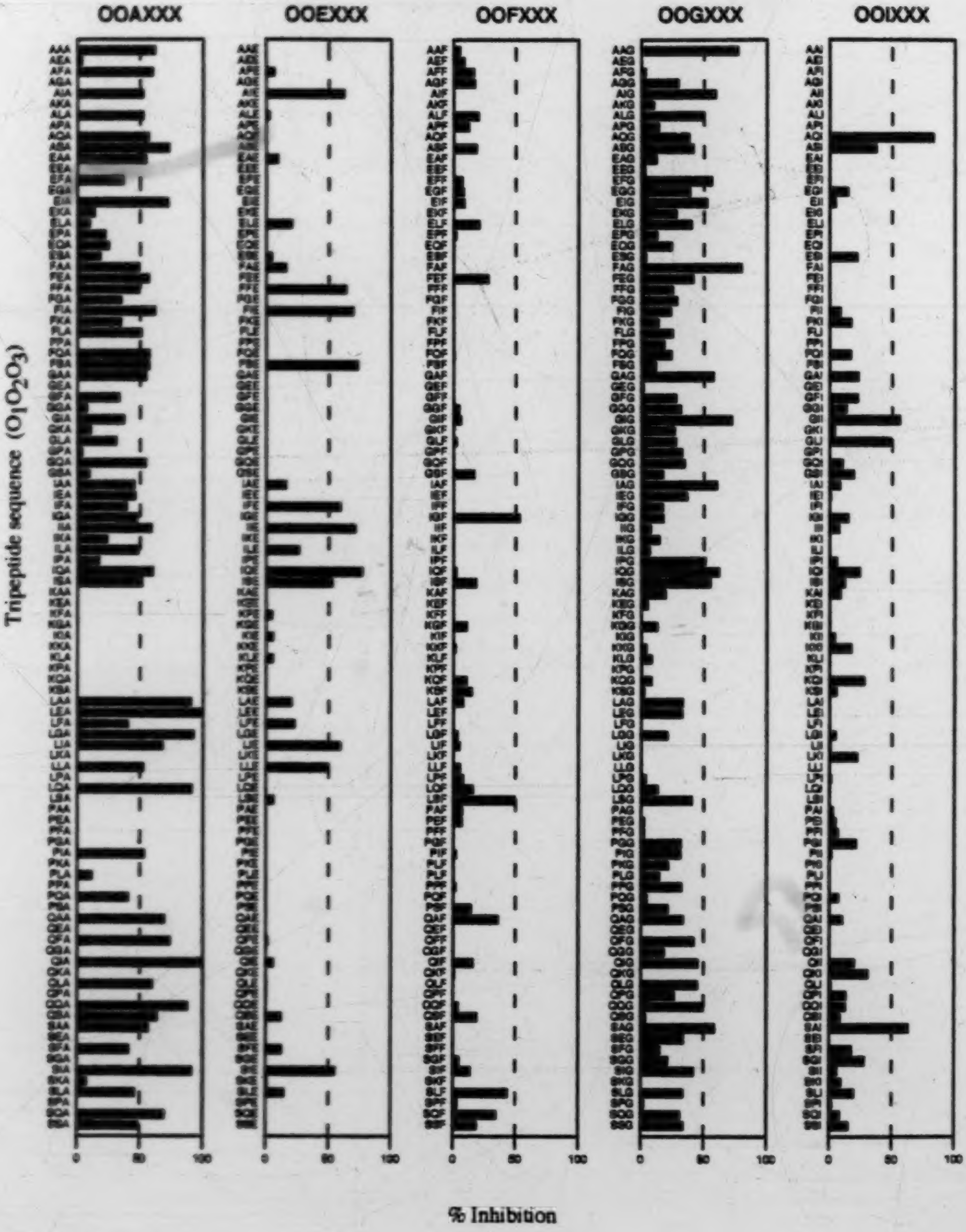


Figure 42 Screening of $O_1O_2O_3X_4X_5X_6$ RPCL (continued) :

Competitive inhibition of EXOS#50 binding to PAK pili

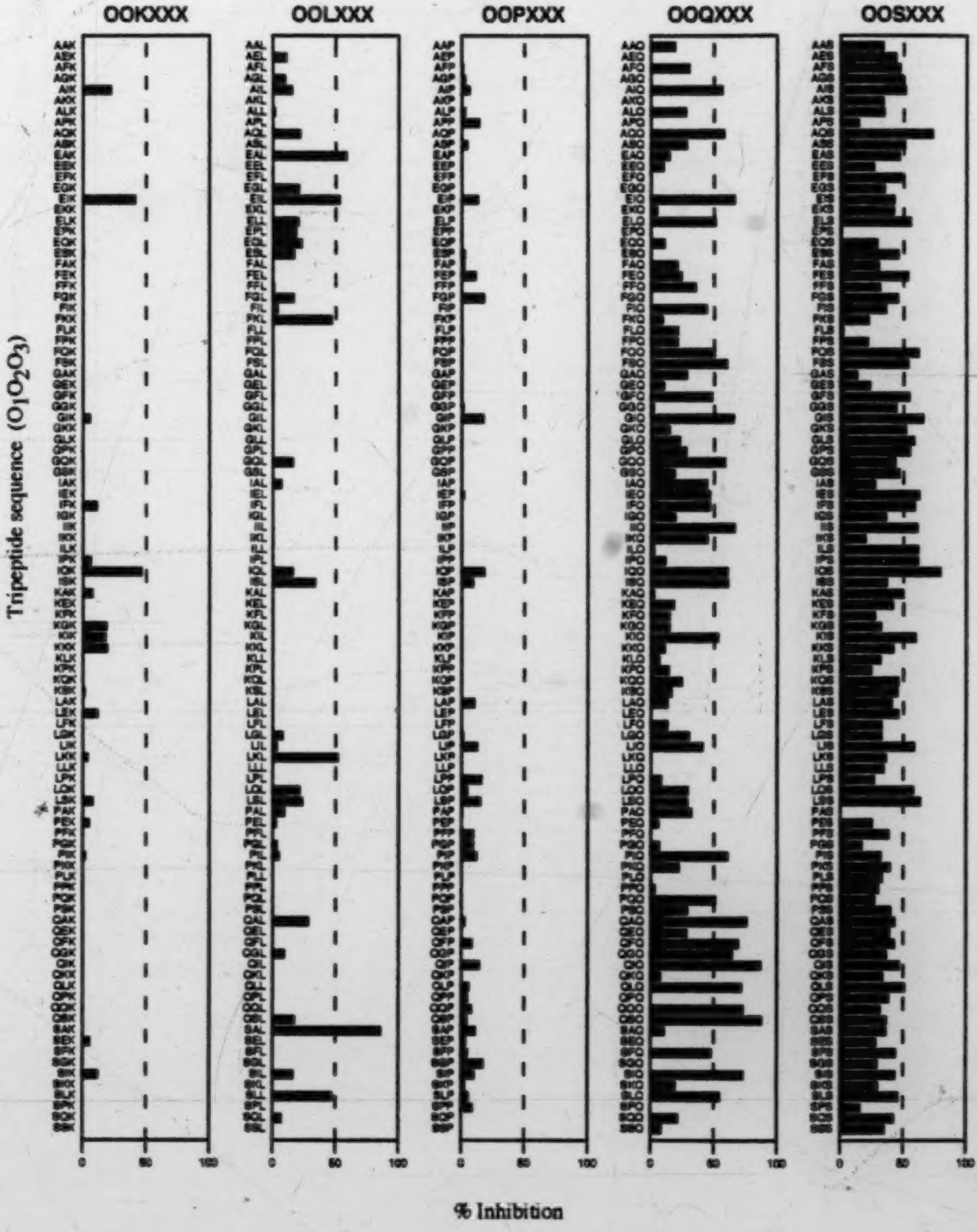
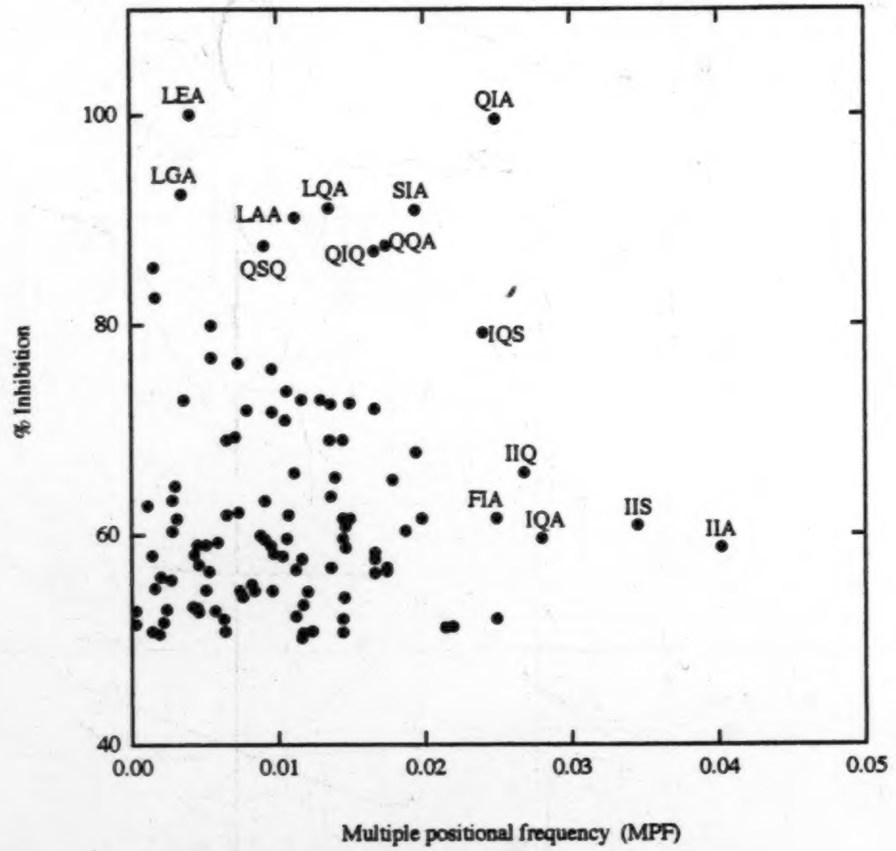


Table 14 Positional Frequency of Representative Amino Acids in Hits From EXOS#50 Initial Screening

Representative amino acid	O_1 frequency	O_2 frequency	O_3 frequency
A	0.18	0.19	0.42
E	0.12	0.07	0.11
F	0.18	0.14	0.02
G	0.15	0.06	0.16
I	0.29	0.33	0.04
K	0.05	0.04	0.01
L	0.14	0.19	0.06
P	0.03	0.03	0
Q	0.18	0.23	0.28
S	0.14	0.18	0.36

Figure 43 **Selection of Triple-Defined RPCL Best Hits for Deconvolution**



Multiple hits obtained in first-round screening of triple-defined RPCL (competitive inhibition of EXOS#50 binding to PAK pilin) are differentiated on the basis of their multiple positional frequency.

Figure 44 **Screening of $O_1O_2O_3O_4X_5X_6$ RPCL :**

Competitive inhibition of EXOS#50 binding to PAK pili

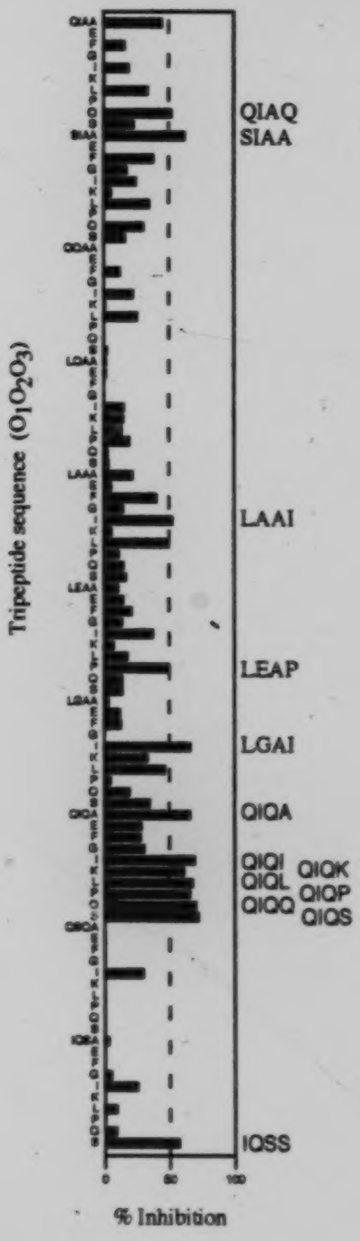


Figure 45 Screening of $O_1O_2O_3O_4O_5X_6$ RPCL :
Competitive inhibition of EXOS#50 binding to PAK pili

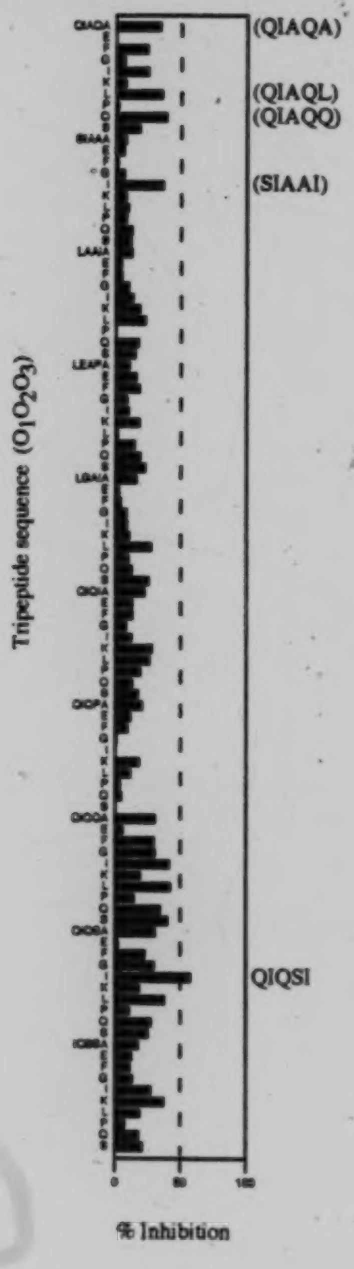
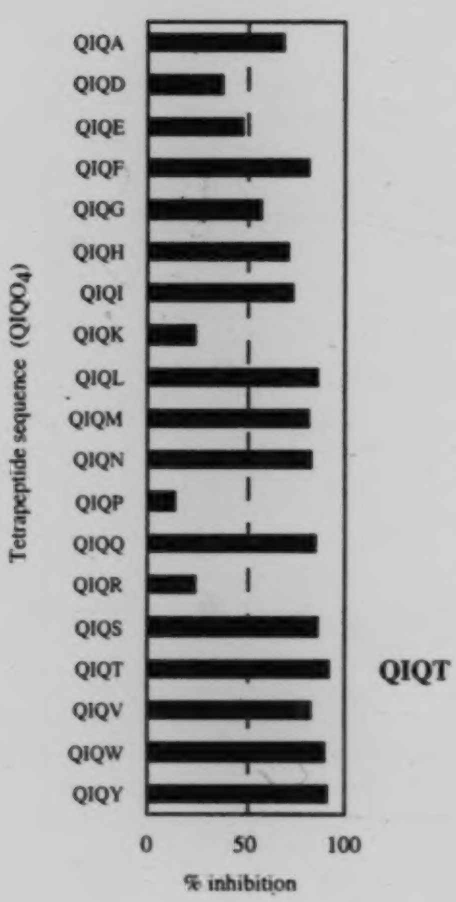


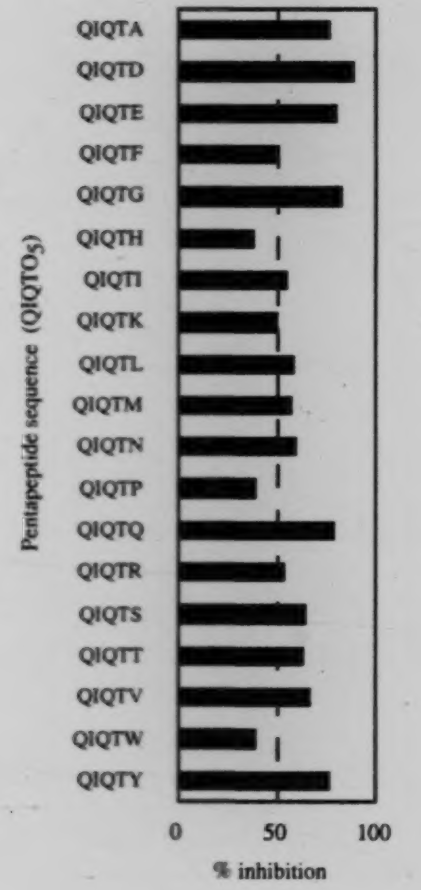
Figure 46 **Screening of QIQO$_4$XX SPCL :**
Competitive inhibition of EXOS#50 binding to PAK pili



Complete set of amino acids (omitting Cys) used in the generation of the sublibrary.

Figure 47 **Screening of QIQTO$_5$X SPCL :**

Competitive inhibition of EXOS#50 binding to PAK pili



Complete set of amino acids (omitting Cys) used in the generation of the sublibrary.

Chapter 6 PEPTIDE PURIFICATION BACKGROUND

6.1 Conventional Peptide Purification

While an efficient peptide synthesis generates only a small number of peptide impurities, the resulting deletion, termination, and/or chemically modified peptides usually possess structures closely related to the peptide of interest and so pose difficult problems in purification. The favoured method of purification for peptides is unquestionably RP-HPLC (reversed-phase high performance liquid chromatography) due to its excellent resolving power, rapid separation times, and the availability of volatile mobile phases; RP-HPLC offers the greatest scope amongst HPLC techniques for manipulation of stationary and mobile phases to improve peptide separation, including choice of packing material and stationary phase functional groups (Esser and Unger, 1991), mobile phase pH and ion-pairing reagent (Bennett, 1991), organic modifier, flowrate and gradient rate (Burke *et al.*, 1991a), and even temperature (Guo *et al.*, 1986). Peptide separation is based on relative overall hydrophobicity, which governs peptide interactions with a strongly hydrophobic stationary phase and determines peptide rate of travel through the column. The stationary phase most frequently consists of straight-chain alkyl C_8 or C_{18} ligands bound to a silica support with a 300 Å pore size matrix and a 5-10 μ particle size range, and is normally maintained at pH 2 to suppress ionic interactions due to silanol ionization (Bij *et al.*, 1981). Unlike smaller organic molecules, peptides partition to only a limited extent, and their interactions with the stationary phase approach an adsorption/desorption mechanism due to multi-site binding (Hodges and Mant, 1991). The aqueous mobile phase employed to carry the sample through the column contains an ion-pairing reagent (e.g. 0.05 - 0.1% TFA for peptide basic groups at pH 2) to enhance sorption and maximize differences in peptide polarities; an organic modifier, normally acetonitrile although isopropanol and methanol are also frequently used (Hermodson and Mahoney, 1983), is added to elute peptides from the column. It is important to note that sample components bind more strongly to the stationary phase than does the eluent, with the possible exception of certain additives in the eluent such as ion-pairing reagents; this is overcome by conditioning the column before sample injection to allow any such additive to reach its equilibrium distribution between the stationary and mobile phases. Flowrates of 1 mL/min, 0.2 mL/min, and 0.1 mL/min are typically employed for analytical (4.0-4.6 mm ID), narrowbore (2-3 mm ID), and microbore (≤1 mm ID) columns respectively, to accommodate the diminishing column internal diameter and produce similar resolution and run times; the advantages of employing smaller diameter HPLC columns include increased sensitivity and reduced solvent consumption (Burke *et al.*, 1991b). Two elution modes are used: isocratic elution, in which the mobile phase composition is kept constant, and gradient elution, which employs a programmed gradual increase in organic modifier concentration. The advantage of increasing elution strength with time lies in reducing the retention times of strongly-sorbed peptides with only a negligible trade-off in resolution (due to the very high resolving power of RP-HPLC). Typically, a linear gradient of 0.5 - 2.0% acetonitrile per minute is employed.

Despite its widespread use, there are disadvantages to the elution mode of RP-HPLC which become increasingly important with scale-up of sample load. Elution mode partially reduces the full extent of the potential separation in the reversed-phase column, as the organic modifier introduced into the mobile phase increases the adsorption/desorption rate. Analytical applications are performed under conditions ensuring linear elution, which requires sufficiently small sample size with respect to the amount of stationary phase in the column to ensure that sample component equilibrium distribution between stationary and mobile phases follows a linear law over the concentration range. There are numerous benefits to adhering to linear elution: this mode of HPLC is well understood theoretically and provides retention times independent of concentration, prevents components from interfering with each other's adsorption (i.e. non-competitive adsorption), and allows components to be separated as distinct symmetrical peaks of approximately Gaussian shape. Unfortunately, the requirements for linear elution result in relatively poor utilization of both stationary and mobile phases. Furthermore, larger-scale linear elution separations require increasingly larger column volumes with correspondingly expensive quantities of packing in order to maintain satisfactory sample loads and product yield, and large columns have very high operating costs per unit of purified product due to high solvent consumption (namely, organic modifier). Even if purification is limited to columns of analytical scale (50-250 mm x 4.0-4.6 mm ID) or semi-preparative scale (250 mm x 7.5-10 mm ID) under conditions of linear gradient elution, a number of runs with relatively low sample loads are required to obtain the desired product yield due to inefficient utilization of column capacity. A large number of fraction analyses may be necessary to locate the peptide product(s) of interest, increasing time and solvent requirements, and product work-up is complicated by the dilution effect of large fraction volumes.

An alternative is the nonlinear displacement mode of RP-HPLC (reviewed in Frenz and Horváth, 1988), which makes significantly more efficient use of the resolving capabilities of reversed-phase packings and thereby allows larger sample loads to be handled by analytical scale columns. In displacement mode, the peptide sample is applied to the column in a carrier solvent with low affinity for the stationary phase. After sorption of the sample near the column inlet, a displacer with greater affinity for the stationary phase than any sample component is slowly pumped onto the column. Sample components displaced from the stationary phase move down the column preceding the displacer front, forming adjacent zones or bands of purified peptides traveling at the same velocity in a "displacement train"; the components may be concentrated over their sample level during displacement. However, the advantage of higher sample load is offset by difficulties in optimizing the choice of displacer, displacer concentration and flowrate, and often very long run times are observed as the result of the low flowrates required (i.e. 0.1-0.2 mL/min). Moreover, this mode requires column regeneration, an operational procedure which makes no contribution to the separation.

In both elution and displacement modes of RP-HPLC some separation of sample components occurs during loading, i.e. the least retained component moves ahead of the other components, and under overload conditions is concentrated to a level greater than in the sample due to the presence of more strongly bound components. Frontal chromatography (Tiselius, 1943) takes advantage of this "displacement effect" by employing continuous sample feed to recover a pure fraction of the least retained component before breakthrough of the other sample components. Such nonlinear nonelution modes of chromatography have desirable features suitable for exploitation in large-scale separations.

6.2 Peptide Purification by Sample Displacement Mode of HPLC

The need for simpler, more efficient and reliable methods of semi-preparative to preparative scale purification has led to the development of a novel method of RP-HPLC suitable for analytical-scale columns, in which the main separation process takes place in the absence of organic modifier or displacer (Burke *et al.*, 1988; Hodges *et al.*, 1991). In the sample displacement mode (SDM) of RP-HPLC, high sample loading in a 100% aqueous mobile phase results in competition by sample components for adsorption sites on the stationary phase and subsequent solute-solute displacement; the sample components act as their own displacers, where the more hydrophobic components compete more successfully and displace the less hydrophobic components from the column. Consequently, this method makes use of the general principles of displacement chromatography without requiring the addition of displacer. Subsequent treatment of the column with an aqueous organic eluent is required only to wash retained components off the column, and makes no contribution to the major separation process. This mode of operation, a hybrid scheme of frontal chromatography followed by elution, is characterized by a marked reduction in solvent consumption, minimal elution volumes, and the collection of far fewer fractions for product isolation than in RP-HPLC (with consequent reductions in time and handling). Potential disadvantages include the requirement for peptide solubility in water (which restricts the range of peptide hydrophobicity which can be accommodated) and the need to determine elution solvent composition before purification (necessitating trials before each new purification).

Most research has been carried out with a model synthetic system composed of five closely related decapeptide analogues S_1 - S_5, designed to simulate a crude peptide mixture typically produced by solid-phase peptide synthesis (which may contain hydrophilic and/or hydrophobic peptide impurities) and to test the efficacy of SDM RP-HPLC in purification. The sequences of these analogues are presented in **Table 15**, where variations in sequence are highlighted within the box. There is only a slight increase in peptide hydrophobicity between S_2 and S_5, reflecting the change from an α-H to a β-CH_3 group between S_2 and S_3 (an increase of only one carbon atom), the change from a β-CH_3 group to two methyl groups attached to the β-CH group between S_3 and S_4 (an increase of two carbon atoms), and the change from an α-H to an α-isopropyl group between S_4 and S_5 (an increase of three carbon atoms). The potential of sample displacement chromatography (SDC) can be seen in simple peptide separations where the desired product is the most hydrophilic or the most hydrophobic component of the mixture (Burke *et al.*, 1988): the former is illustrated in **Figure 48** by separation of a four-peptide mixture (S_2 - S_5) while the latter is illustrated in **Figure 49** by the separation of a five-peptide mixture (S_1 - S_5), where in each case product P and impurity I are identified by subscript. Panel A shows the analytical separation profile of the sample mixture while Panel B shows the SDC elution profile; Peaks I and II were subjected to gradient elution analysis and the results are shown in Panels C and D, respectively. The importance of optimizing sample load to obtain these results must be emphasized. In the case where hydrophobic impurities displace a desired hydrophilic peptide (i.e. classical frontal chromatography), too high a sample load results in contamination with hydrophobic impurities of the product appearing in the breakthrough fraction, whereas too low a sample load results in product remaining on the column (where it is contaminated with hydrophobic impurities). In the case where hydrophilic impurities are displaced off the column by a desired hydrophobic peptide (which in turn remains bound to the column), too high a sample load results in product appearing in the breakthrough fraction (where it is contaminated with the displaced hydrophilic impurities); too low a sample load results in hydrophilic impurities remaining with the product on the column. Separation of a more complex peptide sample containing both hydrophobic and hydrophilic impurities is more problematic: both hydrophilic impurities and product are displaced from the column while hydrophobic impurities are retained (to be removed later by gradient elution), and overlap between the hydrophilic impurity and product zones adversely affects product recovery.

The necessity for optimizing sample load (or alternatively, column length), and poor product recovery from a more complex peptide sample containing both hydrophobic and hydrophilic impurities, led to a multi-column approach in which up to 10 short (3 cm) column segments are joined in series (Hodges *et al.*, 1991). Following SDC, each column segment is isocratically eluted (eliminating the previous gradient wash to recover components retained on the column) and the distribution of sample peptides over the entire column is assessed. This approach is clearly demonstrates the full potential of sample displacement chromatography: the system mimics the separation results of a standard analytical column where each column segment represents a collected fraction (although fraction analysis is greatly simplified by the low number of column segments). Components are distributed on the column according to their relative hydrophobicities, such that the most hydrophilic component is found near the column outlet (segment 1) or in the breakthrough fraction (0) while the most hydrophobic component is found near the column inlet (segment 10). **Figure 50** illustrates the separation of a synthetic decapeptide crude mixture where P is the desired product, I_1 is a hydrophilic impurity, and I_2 - I_5 are hydrophobic impurities; the breakthrough fraction and column segment 1 contain only hydrophilic impurity, column segment 10 contains only the three hydrophobic impurities, and column segments 2 - 7 contain essentially pure product, the great majority of which is found in column segments 3 - 7. This approach improves product recovery and purity, but the column arrangement imposes an undesirable degree of manual manipulation for each sample run and wastes the resolving power of the column. The multi-column approach can be effectively reduced to a "hydrophobic trap" represented by column segments 8-10, a "product isolation column" represented by column segments 2-7, and a hydrophilic fraction represented by the breakthrough fraction and column segment 1.

Refinement of the multi-column approach to the minimal case generated a two-column approach to handle typical crude peptide mixtures containing both hydrophobic and hydrophilic impurities (Mant and Hodges, 1991). As illustrated in **Figure 51**, sample is applied at high load to the two-column system composed of a short pre-column C1 and a main product isolation column C2 in series (Step1); hydrophobic contaminants are retained by the pre-column (the hydrophobic trap), while the desired peptide product moves through the main column displacing hydrophilic impurities. Isolation of the pre-column from the main column removes hydrophobic contaminants while the desired peptide product saturates the main column and displaces hydrophilic impurities off the column (Step 2). Product is recovered from the main column by isocratic and/or gradient elution (Step 3), and the pre-column is reconnected in series to allow hydrophobic impurities to be removed by gradient elution to regenerate the system (Step 4). The size of the pre-column is regulated by the amount of hydrophobic impurities in the sample, while the size of the main column is regulated by the amount of peptide product to be recovered. The use of a pre-column (enabling the operational division of hydrophobic impurity removal and product isolation) ensures that exact knowledge of the sample load is much less critical for successful separation than in the case of a single column. **Figure 52** illustrates the successful separation of the synthetic five-peptide mixture (S_1 - S_5), where product P and impurity I are identified by subscript. Panel A shows the analytical gradient elution profile of the sample mixture while Panel B shows the SDC elution profile; Pools I and II and the pre-column wash were subjected to gradient elution and the results are shown in Panels C, D, and E, respectively. All hydrophobic impurites are retained on the pre-column (along with a small amount of product); all hydrophilic impurities are displaced from the main column in Pool 1 (along with a very small amount of product), and pure product is recovered by displacement and by gradient elution of the main column in Pool II.

Table 15 Sequences of Model System Synthetic Peptides

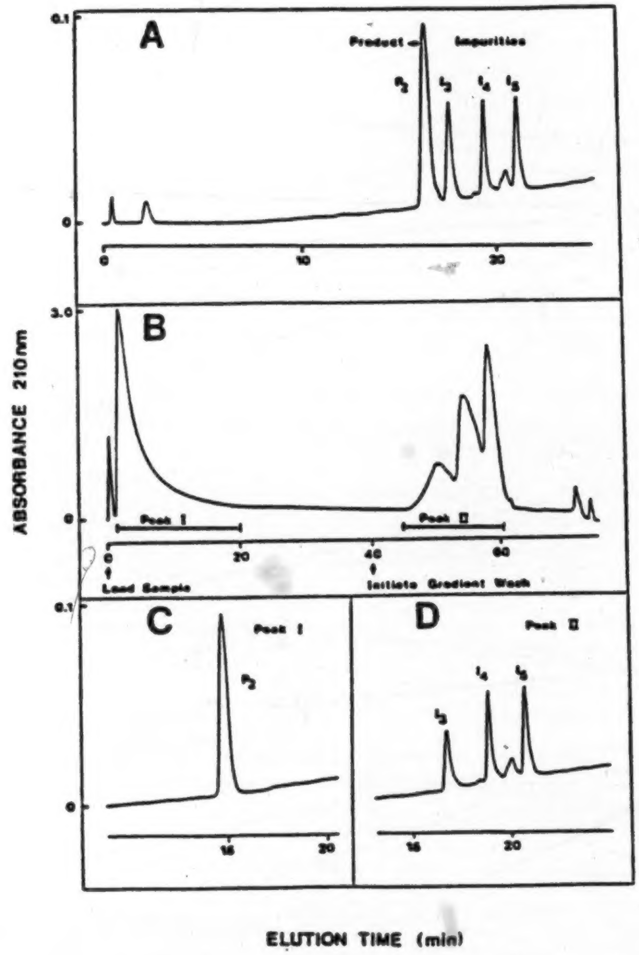
Standard	Sequence
S_1	R G A G G L G L G K -amide
S_2	Ac- R G G G G L G L G K -amide
S_3	Ac- R G A G G L G L G K -amide
S_4	Ac- R G V G G L G L G K -amide
S_5	Ac- R G V V G L G L G K -amide

Ac = N^{α}-acetyl; amide = C^{α}-amide.
Variations in sequence are shown within the box.

Figure 48 **Separation by SDC of peptide product (P_2) from hydrophobic peptide impurities (I_3, I_4, I_5)**

Panel A: Analytical gradient elution profile of sample mixture
Panel B: SDC elution profile of sample mixture
Panel C: Analytical gradient elution profile of Peak 1
Panel D: Analytical gradient elution profile of Peak 2

(Burke *et al.*, 1988)

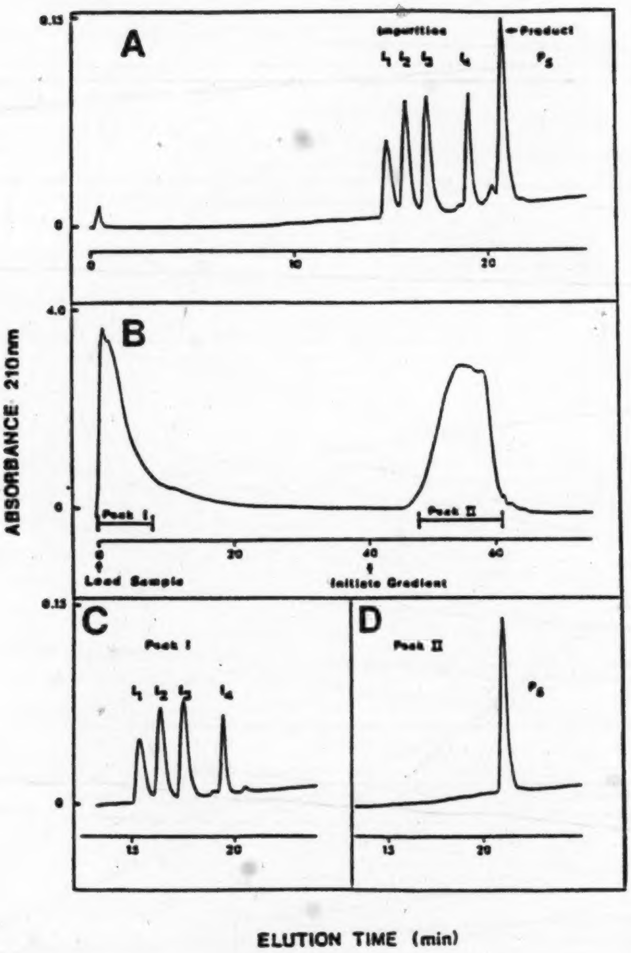


Column: Aquapore RP 300 C_8 (30 x 4.6 mm ID)
Conditions: Panel A: Linear AB gradient (1% B/min) at a flowrate of 1 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile.
Panel B: Isocratic elution with 100% Eluent A for 40 min at a flowrate of 1 mL/min, followed by linear gradient elution at 1% B/min.
Panel C: Same conditions as Panel A.
Panel D: Same conditions as Panel A.

Figure 49 **Separation by SDC of peptide product (P_5) from hydrophilic peptide impurities (I_1, I_2, I_3, I_4)**

Panel A: Analytical gradient elution profile of sample mixture
Panel B: SDC elution profile of sample mixture
Panel C: Analytical gradient elution profile of Peak 1
Panel D: Analytical gradient elution profile of Peak 2

(Burke *et al.*, 1988)

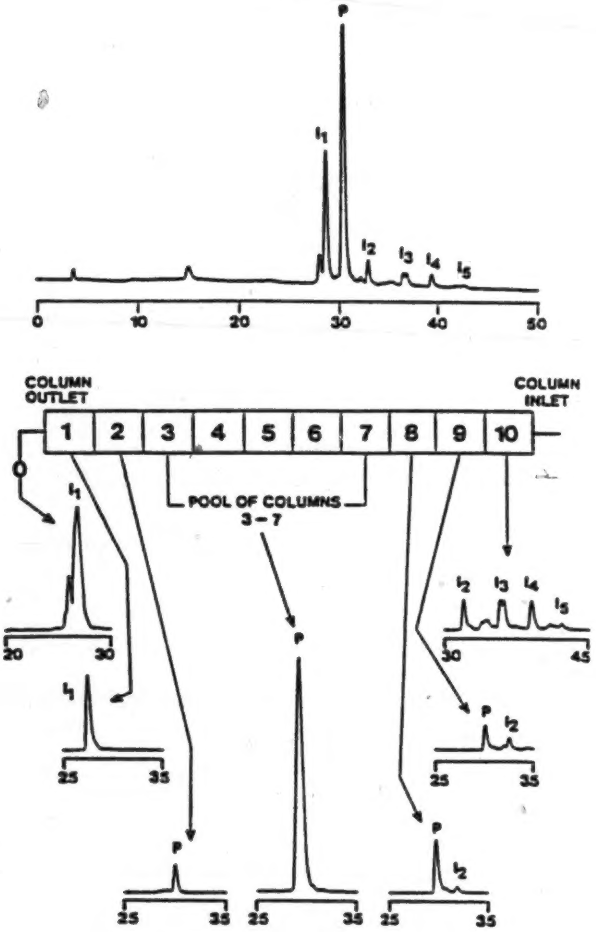


Column: Aquapore RP 300 C_8 (30 x 4.6 mm ID)
Conditions:
Panel A: Linear AB gradient (1% B/min) at a flowrate of 1 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile.
Panel B: Isocratic elution with 100% Eluent A for 40 min at a flowrate of 1 mL/min, followed by linear gradient elution at 1% B/min.
Panel C: Same conditions as Panel A.
Panel D: Same conditions as Panel A.

Figure 50 **Multi-column separation by SDC of a synthetic decapeptide crude mixture**

Top:	Analytical gradient elution profile of crude mixture
Middle:	Schematic of 10-segment column
Bottom:	Analytical gradient elution profiles of peptide components retained on each column segment and breakthrough (0)

(Hodges *et al.*, 1991)

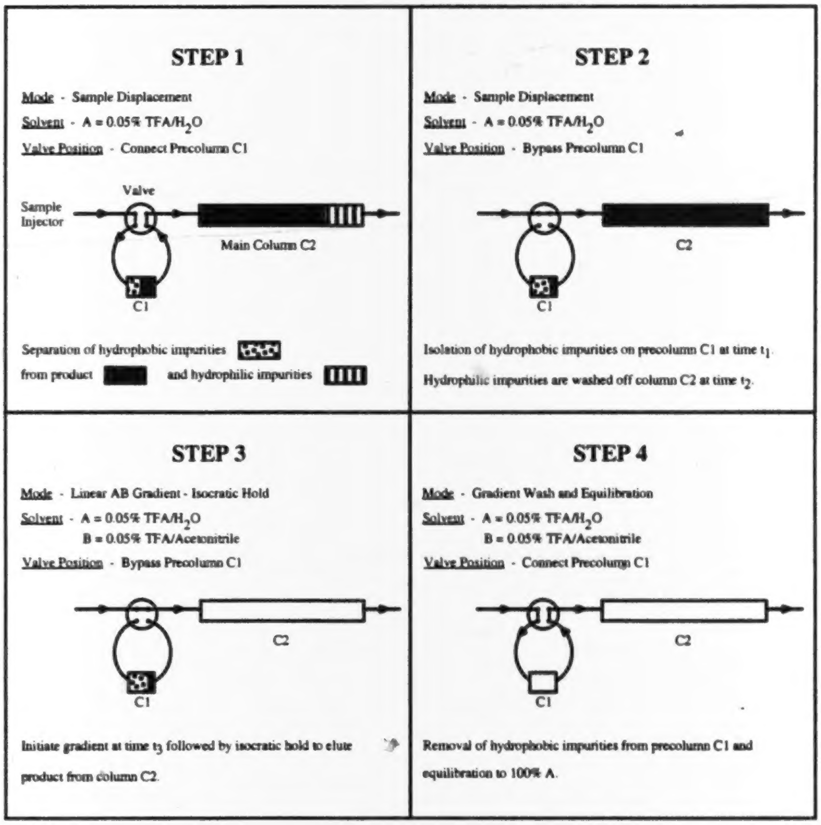


Column: Ten Aquapore RP 300 C_8 (30 x 4.6 mm ID) column segments in series

Conditions:
Top: Linear AB gradient (1% B/min) at a flowrate of 1 mL/min, Eluent A is 0.1% aqueous TFA and Eluent B is 0.1% TFA in acetonitrile.
SDC: Isocratic elution with 100% Eluent A for 50 min at a flowrate of 0.5 mL/min, followed by isocratic elution of column segments at 25% Eluent A.
Bottom: Same conditions as Top.

Figure 51 Two-Column SDC Approach

(Mant and Hodges, 1991)



STEP 1

Mode - Sample Displacement

Solvent - A = 0.05% TFA/H_2O

Valve Position - Connect Precolumn C1

Separation of hydrophobic impurities ▨
from product ■ and hydrophilic impurities ▥

STEP 2

Mode - Sample Displacement

Solvent - A = 0.05% TFA/H_2O

Valve Position - Bypass Precolumn C1

Isolation of hydrophobic impurities on precolumn C1 at time t_1.

Hydrophilic impurities are washed off column C2 at time t_2.

STEP 3

Mode - Linear AB Gradient - Isocratic Hold

Solvent - A = 0.05% TFA/H_2O
B = 0.05% TFA/Acetonitrile

Valve Position - Bypass Precolumn C1

Initiate gradient at time t_3 followed by isocratic hold to elute product from column C2.

STEP 4

Mode - Gradient Wash and Equilibration

Solvent - A = 0.05% TFA/H_2O
B = 0.05% TFA/Acetonitrile

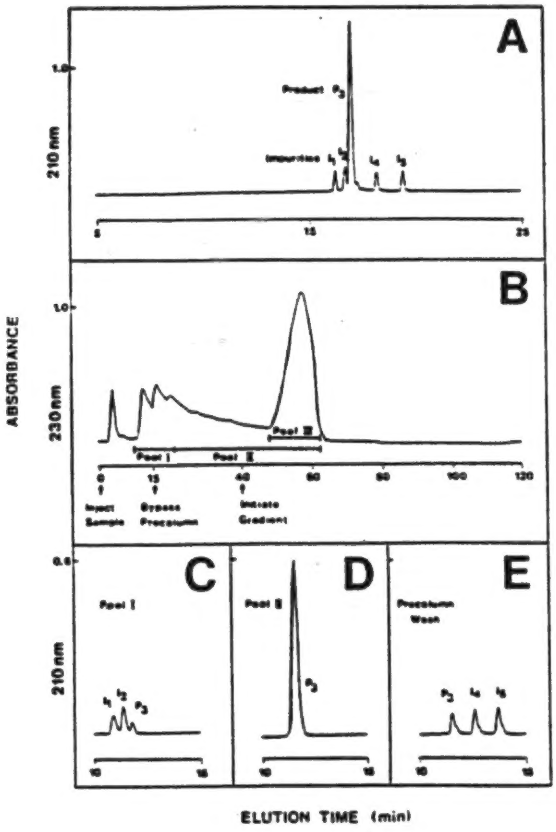
Valve Position - Connect Precolumn C1

Removal of hydrophobic impurities from precolumn C1 and equilibration to 100% A.

Figure 52 **Two-column separation by SDC of peptide product (P_3) from hydrophilic (I_1, I_2) and hydrophobic (I_3, I_4) peptide impurities**

Panel A: Analytical gradient elution profile of sample mixture
Panel B: SDC elution profile of sample mixture
Panel C: Analytical gradient elution profile of Pool I
Panel D: Analytical gradient elution profile of Pool II
Panel E: Analytical gradient elution profile of components retained on pre-column

(Mant and Hodges, 1991)



Columns: Aquapore RP 300 C_8 (4.6 mm ID): analytical, 30 mm length; pre-column, 2 x 30 mm length; main column, 220 mm length.

Conditions:
Panel A: Linear AB gradient (2% B/min) at a flowrate of 1 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile.
Panel B: Isocratic elution with 100% Eluent A for 40 min at a flowrate of 1 mL/min, followed by linear gradient elution at 1% B/min.
Panel C: Same conditions as Panel A.
Panel D: Same conditions as Panel A.
Panel E: Same conditions as Panel A.

6.3 Development of Multiple Peptide Purification

The growing requirement for synthetic peptides in numbers and quantities suitable for large-scale research and development applications has been the stimulus for the development of efficient multiple peptide synthesis, and is now driving the demand for equally rapid and effective peptide purification. The last decade has seen the development of ultrafast HPLC in which peptide and protein separations are accomplished in under 5 minutes; microbore columns packed with non-porous or macroporous supports to reduce mass transfer resistance are able to operate at 5 - to 10 - fold normal flow velocities, and instrumentation optimized with respect to flow dynamics and minimal system volumes ensures rapid equilibration between sample injections. This technology has been designed specifically for on-line analysis of peptide or protein syntheses and is less appropriate for rapid peptide purification, since the need for tedious fraction collection and analysis remains and the reduction in actual separation time becomes less significant as the number of peptides undergoing purification rises. As seen with multiple peptide synthesis, there is a need for simultaneous multiple peptide purification rather than for improvements in the conventional serial approach. This may prove even more of a challenge, combining the need for operational simplicity with rapid purification of large numbers of crude peptides produced in varying yield and displaying a wide range of overall hydrophobicity.

One approach to achieving this goal involves the adaptation of SDC to the operating constraints of a simultaneous purification scheme. The prime consideration is the technical complexity imposed by maintaining equal flow distribution while operating parallel multiple columns at high pressure - clearly, a low pressure operation is desirable for simplicity and reliability. Practical application of SDC is possible only if the benefits of high sample load, aqueous separation in the absence of an organic modifier, and the excellent resolving power of RP-HPLC, are retained under low pressure conditions. Successful development of this multiple purification scheme also requires a low-cost alternative to standard HPLC columns, and a ready-made solution may be found in the application of solid phase extraction (SPE) technology. A convenient, inexpensive, and time-saving alternative to liquid-liquid extraction traditionally used for cleaning and concentrating analytical samples, SPE offers a variety of bonded-phase silica-based packings (including reversed-phase) to extract contaminants or analytes of interest from liquid solution. Disposable polypropylene tubes containing these packings are designed to operate as extraction columns under low pressure conditions where sample solutions are passed through using either positive pressure or partial vacuum, and are ideally suited for adaptation to a low pressure version of SDC. The choice of SPE packed tube size depends on sample volume, amount of analyte of interest (or degree of contamination), strength of analyte/sorbent interaction, sample solvent strength, and sample complexity, factors also important to RP-HPLC. Customized arrangements are made possible by the use of stackable tubes, a range of tube sizes (1-12 mL, containing 100-2000 mg packing), sample reservoirs (20-50 mL capacity), and a variety of collection vessels (including test tubes, 1-10 mL volumetric flasks, autosampler and scintillation vials); most importantly, multiple extractions can be accommodated simultaneously by attaching 12-24 tubes to SPE vacuum manifolds fitted with collection vessel racks, providing a ready-made system for testing the potential of the low pressure chromatographic technique. Two SPE operational modes exist, depending upon the desired product: in clean-up applications, the sample is applied to the tube and analytes of interest are collected once they pass through the adsorbent, while contaminants retained on the packing can be disposed of with the tube; in concentration applications, contaminants pass through the tube while analytes of interest are retained on the packing and are selectively eluted in a small volume of the appropriate solvent to increase their concentration. These two modes of operation are easily adapted to the hydrophobic trapping and product isolation operations of SDC required for peptide product separation from hydrophobic and hydrophilic impurities.

Chapter 7 DESIGN OF A MULTIPLE PEPTIDE PURIFICATION SYSTEM

7.1 Design Proposal

The combination of solid phase extraction (SPE) technology and sample displacement chromatography (SDC) methodology may provide a facile, rapid and cost-effective approach to multiple peptide purification. In the proposed purification system design presented in **Figure 53**, peptide purifications are performed in extraction columns consisting of disposable SPE tubes with silica-based reversed-phase packing. A low pressure version of SDC is achieved by adding crude peptide sample at overload conditions and applying vacuum to draw the solution through the packing. Product separation from hydrophobic and hydrophilic impurities is accomplished in a two-stage purification unit which takes advantage of the two SPE operational modes: a short pre-column is used in a clean-up application, functioning as a trap for hydrophobic impurities, and a second longer column is used in a concentration application, functioning as a product isolation column. Crude peptide sample is loaded onto the purification unit in 100% aqueous solvent at overload conditions to achieve sample displacement mode (SDM) operation, and is washed through trap and product isolation column in sequence. Under ideal SDC conditions, hydrophobic impurities are retained on the trap, product and hydrophilic impurities are displaced from the trap to the product isolation column, and hydrophilic impurities are displaced off the product isolation column to waste; only product is retained on the main column. The product is subsequently removed from this column by isocratic elution with organic modifier, and is recovered from solution by lyophilization. This arrangement reduces the extensive fraction collection and analysis required by analytical RP-HPLC for product isolation to an absolute minimum: only three "fractions" exist, namely the hydrophobic trap, the product isolation column, and the waste wash, and only the retained peptide on the product isolation column is collected. Simultaneous multiple purifications are carried out by mounting tubes on a standard SPE processing station connected to an in-house vacuum line. Lacking pumps, injectors, solvent mixing systems and the like, this purification system design uses minimal instrumentation compared with RP-HPLC, and avoids the need for high pressure containment.

Performance objectives of the two-stage purification unit are summarized in **Figure 54**, and are based on sacrificing some product yield to ensure product purity. The purification unit is designed to handle sample loads of up to 10 mg crude peptide and to recover at least 80% of the product, with no more than 10% losses to each of the hydrophobic trap and waste wash and the corresponding removal of essentially all hydrophobic and hydrophilic impurities in these respective fractions.

Figure 53 Schematic of Multiple Peptide Purification System

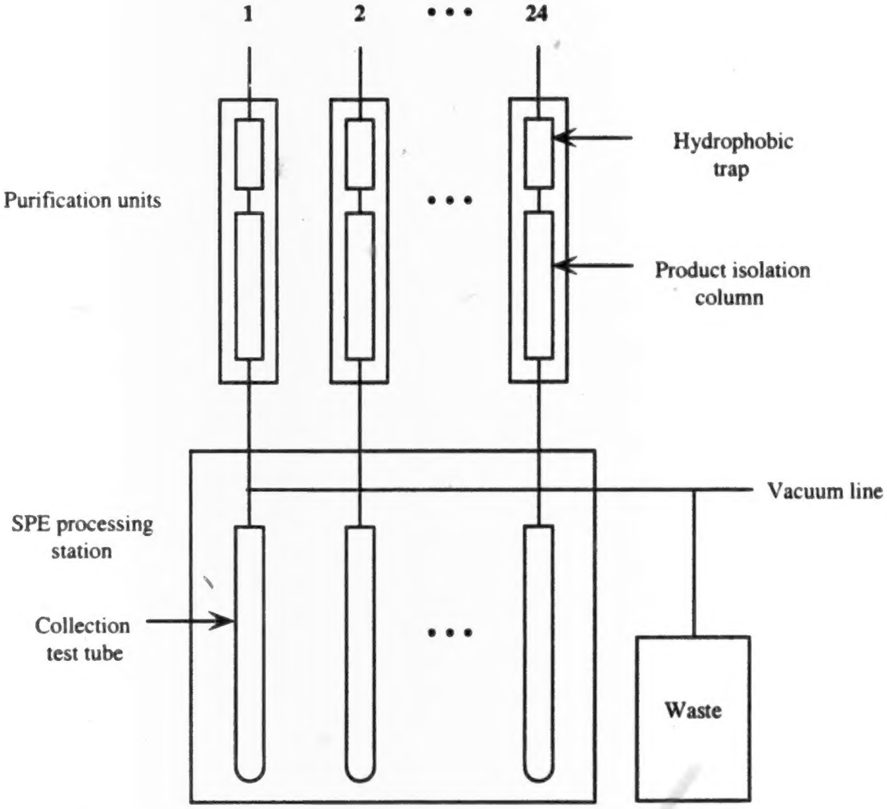
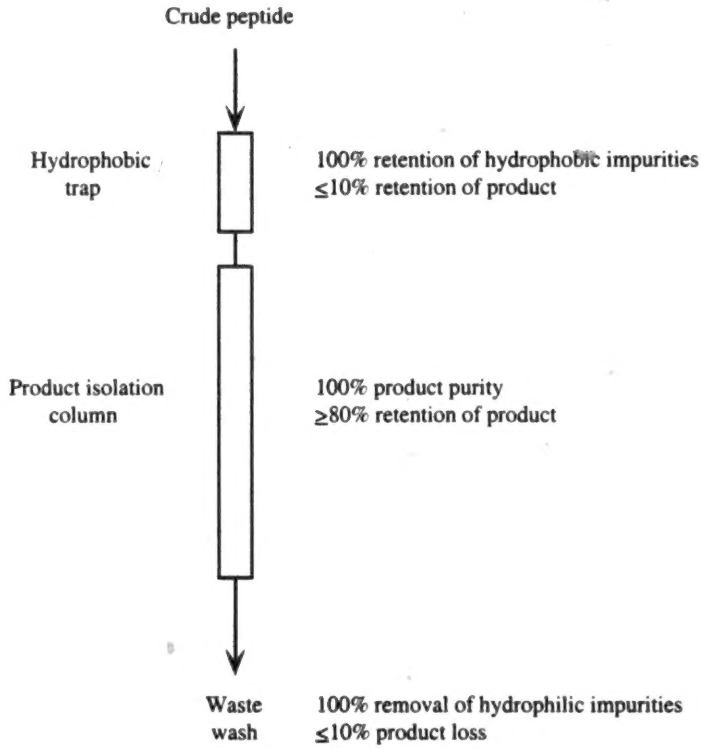

1
2
• • •
24
Hydrophobic trap
Purification units
• • •
Product isolation column
Vacuum line
SPE processing station
Collection test tube
• • •
Waste

Figure 54 Performance Objectives of Two-Stage Purification Unit


Crude peptide
Hydrophobic trap
100% retention of hydrophobic impurities
≤10% retention of product
Product isolation column
100% product purity
≥80% retention of product
Waste wash
100% removal of hydrophilic impurities
≤10% product loss

7.2 Purification System Development

The major challenge faced by this multiple peptide purification system lies in the variable nature of the crude peptides undergoing purification. Peptides show significant variation in overall hydrophobicity, and may be synthesized at various scales and exhibit a range of purity. For routine operation of a simultaneous purification system, it is necessary to use a standardized purification unit with the appropriate operating protocol(s) to achieve a specified product purity for a range of peptide hydrophobicities, crude loads and impurities.

Purification Unit

The development of a standardized purification unit was based on sample loads of up to 10 mg in solution volumes of 1-5 mL, leaving larger loads to be handled by additional runs. Following the rule of thumb that sample size should be 5% of the packing bed (i.e. a 100 mg bed should retain 5 mg of sample), a 1 mL SPE tube designed to hold 100 mg of RP-packing and 1 mL sample volume was selected for use as both hydrophobic trap and product isolation column; the stackable nature of these tubes ensured a "building block" approach to development, should longer columns be required, and larger solution volumes could be accommodated using 20 mL sample reservoirs. The SPE tubes were filled manually with variable quantities of reversed-phase packing sandwiched between polyethylene frits during developmental work; column size was reported as mg packing, since constant column diameter was maintained. Trap and product isolation column were joined in series by means of an adapter to form a two-stage purification unit **(Figure 55)**. Purification units were mounted on a 24-position vacuum-operated Vac Elut SPS24 SPE processing station (Analytichem International, USA) equipped with stainless steel sample delivery tips and a 13x100 mm test tube collection rack **(Photograph 16)**.

Operating Procedures

Preparation of the two-stage purification unit entails packing and conditioning of the column and assembly of the unit (outlined in **Figure 56**), while unit operation proceeds in discrete stages of sample loading, washing, and elution (summarized in **Figure 57**). Packing beds should not be allowed to dry out between stages of operation, nor should very large sample volumes be used since RP-packings gradually lose the solvent layer acquired through conditioning when excessive volumes of aqueous solutions are extracted. As described in **Table 16**, a manual packing procedure was developed for SPE tubes to produce trap and product isolation columns of variable packing bed length. Conditioning, sample loading, washing, and elution procedures were drawn from SPE methodology with adaptations from SDC in terms of solvent selection: sample loading and washing were performed with water, while elution was carried out with 50% acetonitrile in water (the level of organic modifier chosen to ensure recovery of all peptide components from trap and column). SPE operational procedures recommend slow sample passage (described as dropwise flow) for greatest extraction; for operation on the VacElut SPE processing station, this was translated into a vacuum-assisted flowrate of approximately 0.7 mL/min, slightly slower than the standard 1 mL/min flowrate used in RP-HPLC applications. A minimal trap wash into the main column was employed to ensure complete passage of the sample liquid (and unretained peptide components) through the trap, before disconnecting the trap from the column and continuing to wash the column. Trap and column wash volumes have been normalized in terms of trap and column volumes (V_{trap} and V_{col}), defined later. The assembled purification unit was subject to an operational constraint under vacuum, arising from the sizeable dead space between the packing beds of the trap and product isolation column: bed solvation in the product isolation column could only be maintained during sample transport through the trap by introduction of an aqueous solvent layer (approximately 350 μL) above the column bed before sample addition to the unit.

Synthetic Peptide Standards

The synthetic peptide standards S_2, S_4, and S_5 were chosen to evaluate performance during development of the purification system, and their sequences are repeated in **Table 17**. As stated previously, these standards are quite closely related in hydrophobicity so as to provide a reasonable challenge to separation - between S_2 and S_4 there is an increase of 3 carbon atoms, and the same increase exists between S_4 and S_5. Lys and Arg residues have been incorporated to provide net positive charge and improve peptide solubility. A mixture of these standards provides a simple represention of a crude peptide produced by solid-phase synthesis, in which a desired product (S_4) is contaminated by single hydrophilic (S_2) and hydrophobic (S_5) impurities **(Figure 58)**.

Packing

Compared with typical silica-based RP-HPLC packings used for peptide/protein separations (characterized by a 300 Å pore size and a particle size range of 5-20 μm), RP-SPE packings possess a larger particle size (to enable fast drainage under positive pressure or partial vacuum conditions) and a smaller pore size (suitable for typical applications involving the extraction of smaller organic molecules). The peptide capacities of a selection of C_8 and C_{18} RP-HPLC packings and a RP-SPE packing (described in **Table 18**) were compared by loading 5-7 mg of synthetic peptide standard S_5 onto single-column purification units containing 100 mg of each packing, washing the columns with a single tube volume (i.e. 1 mL) of 0.05% aqueous TFA, and eluting peptide with a single tube volume of 0.05% TFA / 50% acetonitrile in water (results presented in **Figure 59**). Since the synthetic standards are small peptides, the reduced pore size of the RP-SPE packing had no adverse effect on peptide capacity.

There was no apparent correlation between peptide capacity and packing reversed-phase ligand (C_8 vs C_{18}), particle size, or pore size **(Figure 60)**, although probable differences between packings of the reversed-phase ligand density (or loading) of the particles could not be taken into account since this proprietory data was not available from all manufacturers. However, the high loading (and hence most hydrophobic nature) of the Kromasil packing undoubtedly prevented sufficient wetting under the conditions employed; the problem could have been overcome by sample addition in 1% aqueous CH_3CN for best results, but this would have contravened the minimalistic conditions of SDC. As expected, particle size had a noticeable effect on sample flowrate through the column, but the SPE processing station was able to compensate for this effect by increasing the vacuum to maintain a constant flowrate of approximately 0.7 mL/min, slightly below the standard 1 mL/min used in HPLC applications. The Rainin and SynChrom RP-HPLC packings exhibited the highest peptide capacities under the conditions employed (6.0 mg /100 mg packing and 5.0 mg/100 mg packing, respectively); the Rainin packing was retained for further developmental work on the basis of highest capacity (under the given conditions), available supplies, and direct comparison with analytical RP-HPLC purification (since only the Rainin packing falls into the normal particle size range used in analytical RP-HPLC).

Operational Parameters

Successful operation of the purification unit depends on three parameters: 1) an optimized trap-to-column packing ratio (to remove all hydrophobic impurities in the trap with minimal product loss and to ensure only the separation of product from hydrophilic impurities occurs within the product isolation column); 2) an optimized wash volume (sufficient for washing out all hydrophilic impurities from the product isolation column while retaining most of the product); and 3) a minimal elution volume for the product isolation column. The effects of wash and elution volumes were investigated using 7 mg sample loads of S_5 peptide standard on a column containing 100 mg of Rainin packing. Peptide was slowly but steadily washed off the column as increasing volumes of wash were applied, demonstrating that product will be released from the packing even in the absence of a more hydrophobic displacer and proving the need to optimize wash volume in order to retain as much product as possible while still displacing all hydrophilic impurities **(Figure 61)**. The effect of increasing elution volume on peptide recovery was negligible - only a small volume of eluent (1 mL of 50% acetonitrile in water) was required to remove all remaining peptide from the column, proving the minimal solvent requirements of this technique. The data also confirm an acceptable degree of accuracy for peptide determination by quantitative HPLC, since the total peptide load applied to the column was recovered from wash and eluent.

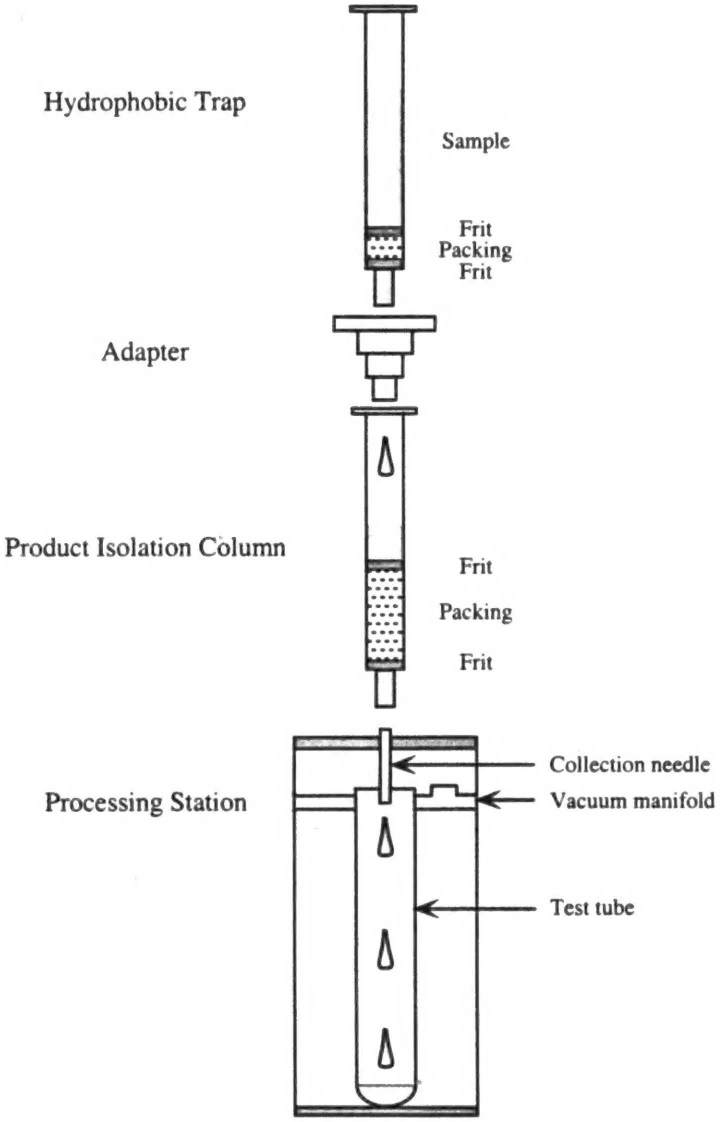
Working on the basis that trap size is determined by the quantity of hydrophobic impurities to be removed and product isolation column size by the quantity of product to be recovered, the initial estimate of trap-to-column packing ratio (outlined in **Table 19**) was based on previously described performance objectives and a 10 mg trap as the minimum acceptable for practical application. Wash and elution volumes were normalized as numbers of trap volumes (V_{trap}) and column volumes (V_{col}) to account for potential differences in scale, based on the total volume of the respective solvated packing beds (rather than the respective void volumes). This simple and rapid method avoided the need to measure void volumes of individual columns by using a packing volume constant (estimated as 205 μL/100 mg solvated Rainin packing in 0.05% aqueous TFA), and provided sufficient accuracy considering the variation encountered in manually packed columns.

Trap size was investigated using a 9:1 mixture of product S_4 to hydrophobic impurity S_5 (as determined by HPLC chromatogram peak area) at a sample load of 4 mg (slightly under the 5-10 mg design range to test scale-up at a later time), and the results are presented in **Figure 62** as % recovered peptide located in the trap. Only 68% of the hydrophobic impurity S_5 was retained by the 10 mg trap, indicating the need for a larger trap with excess capacity to allow S_5 displacement by product S_4 to proceed. A 1.5-fold increase in trap size to 15 mg packing permitted complete retention of the hydrophobic impurity, and the concomitant product loss of 14% constituted a negligible increase over the 11% product loss in the 10 mg trap. The importance of a trap wash (a minimal volume of 5 V_{trap} = 155 μL) into the product isolation column to ensure complete passage of the sample liquid and unretained sample components was clearly demonstrated: a minimal wash of the 10 mg trap lowered the retained product in the trap from 17% to 11%, corresponding to a 35% reduction in product loss (6% / 17%) with virtually no change in hydrophobic impurity retention, and a wash of the optimally sized 15 mg trap provided an even more dramatic 46% decline in product loss (12% / 26%, corresponding to the decline in retained product on the trap from 26% to 14%) while completely retaining the hydrophobic impurity. The performance of the complete purification unit with optimized trap is presented in **Figure 63**, where the wash fraction is so-named due to the absence of hydrophilic impurities in the two-component sample. An 18% loss of product to the wash fraction was observed, suggesting a need to increase the size of the product isolation column.

Sizing of the product isolation column was investigated using a 1:9:1 mixture of hydrophilic impurity S_2 to product S_4 to hydrophobic impurity S_5 (i.e. retaining the previous ratio of S_4 to S_5, and adding S_2 to the sample in the same ratio as the hydrophobic impurity to examine washout demands on the column); results are presented in **Figure 64**. The 15 mg trap selected during trap sizing was successful at achieving the performance objectives with the new three-component sample mixture, removing 100% of the hydrophobic impurity with only 14% product loss. The importance of a trap wash was once again clearly illustrated with the 56 mg product isolation column: 16% of the hydrophilic impurity remained in the trap in the absence of a trap wash and hydrophilic impurity contaminated the product isolation column, due to incomplete passage of the sample liquid through the trap. The trap wash also cut product loss on the trap in half (from 33% to 14%, close to the 10% performance objective) by extending S_5 displacement of S_4, and this product in turn displaced all hydrophilic impurity from the product isolation column. In the process of achieving complete hydrophilic impurity removal from the 56 mg column, an 11% product loss to the wash was observed (essentially achieving the 10% performance objective), and the 74% product recovery was within reach of the performance objective of 80%. As seen in **Figure 65**, lengthening the product isolation column improved product recovery, although an almost 2.25-fold increase in column size (from 56 mg to 125 mg packing) achieved only a 1.12-fold increase in product recovery (from 74% to 83%). The unexpected presence of approximately 15% S_5 in the 100 mg and 125 mg columns suggests that too large a wash (i.e. more than the designated 5 V_{trap}) was used on the two 15 mg traps before disconnecting them from their product isolation columns, or possibly a slightly smaller amount of packing was present in these traps. Complete removal of hydrophilic impurity appeared possible provided sufficient wash volume was used (in the case of the 100 mg column size, S_2 remained in the column due to incomplete washthrough of S_4 and the corresponding displacement of S_2). Acting on the assumption that the length of the product isolation column could be manipulated to achieve complete removal of the hydrophilic impurity in a reasonable volume of wash before the first loss of product, thereby maximizing both product purity and product recovery, the patterns of hydrophilic impurity and product washout over the given range of column size were monitored and have been presented in **Figure 66**. In the case of the smallest column (i.e. 56 mg packing), 9% of the hydrophilic impurity emerged with the sample solution in the absence of a column wash, and product loss from the column occurred almost immediately (a 4% loss with the application of a wash volume of only 2 V_{col}). The breakthrough of S_2 and its steep rate of removal indicated the hydrophilic impurity had been displaced to the end of the column by product during sample loading, and only minimal washing (7 V_{col}) was required to finish the separation. This represents the minimum column required for purification. The hydrophilic impurity was concentrated by a factor of 1.33-fold greater than in the sample (having been applied in 1 mL and collected in 0.75 mL), illustrating a feature of frontal chromatography. The gradual rate of product loss reflects adsorption/desorption down the column in the absence of a stronger displacer component. Increasing the column size beyond the minimum (from 75 mg to 125 mg) resulted in a much more gradual and roughly constant rate of removal of the hydrophilic impurity in the wash as S_2 distributed through the column was driven out by the product, and subsequent development with a sufficiently long column (i.e. 100 mg) of a lag before S_2 emergence in the wash. Run times were relatively short, as all hydrophilic impurity was removed from even the 125 mg column after 60 V_{col}, corresponding to a run time of approximately 22 minutes at an average flowrate of 0.7 mL/min. Choosing a trap-to-column packing ratio greater than 15:75 did not provide a greater degree of separation within the product isolation column for the three-component sample mixture, but a larger ratio may be beneficial when dealing with more complex sample mixtures (requiring the separation of more than two components in the column) or with less hydrophobic sample mixtures (which would pass more rapidly through the column and might not achieve the desired degree of separation before emerging in the wash). On this basis, the 15:100 trap-to-column packing ratio was chosen for future work.

The ability to scale trap and column sizes for sample load based on maintaining a constant trap-to-column packing ratio was tested using 5 mg and 10 mg loads of the 1:9:1 three-component sample mixture (corresponding to 1.25- and 2.5-fold increases in load). This basis for scale-up from the original 4 mg sample load was very successful, as the 15:100 trap-to-column packing ratio provided nearly identical system performance when trap and column sizes were linearly increased to 19:125 and 38:250 for the 5 mg and 10 mg sample loads, respectively **(Figure 67)**. Product recovery was maintained at 82-84%, with trap and wash losses of 9-11% and 7-8% respectively, and in each case all hydrophobic impurity was removed by the trap. The amount of washing required to achieve this performance was similar: the 10 mg load required a 1.4-fold greater proportionate wash (an additional 19 V_{col}). This reflected a somewhat slower rate of S_2 removal from the larger column as seen in **Figure 68**; in addition, the lag before S_2 emergence in the wash was less well defined.

Figure 55 Schematic of Two-Stage Purification Unit



Hydrophobic Trap
Sample
Frit
Packing
Frit
Adapter
Product Isolation Column
Frit
Packing
Frit
Processing Station
Collection needle
Vacuum manifold
Test tube

Photograph 16 VacElut SPS24 SPE processing station

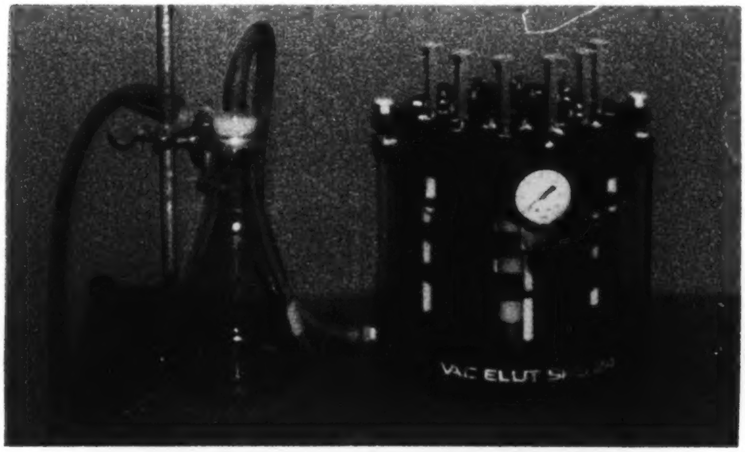

VAC ELUT SPS24

Figure 56 Preparation of Purification Unit

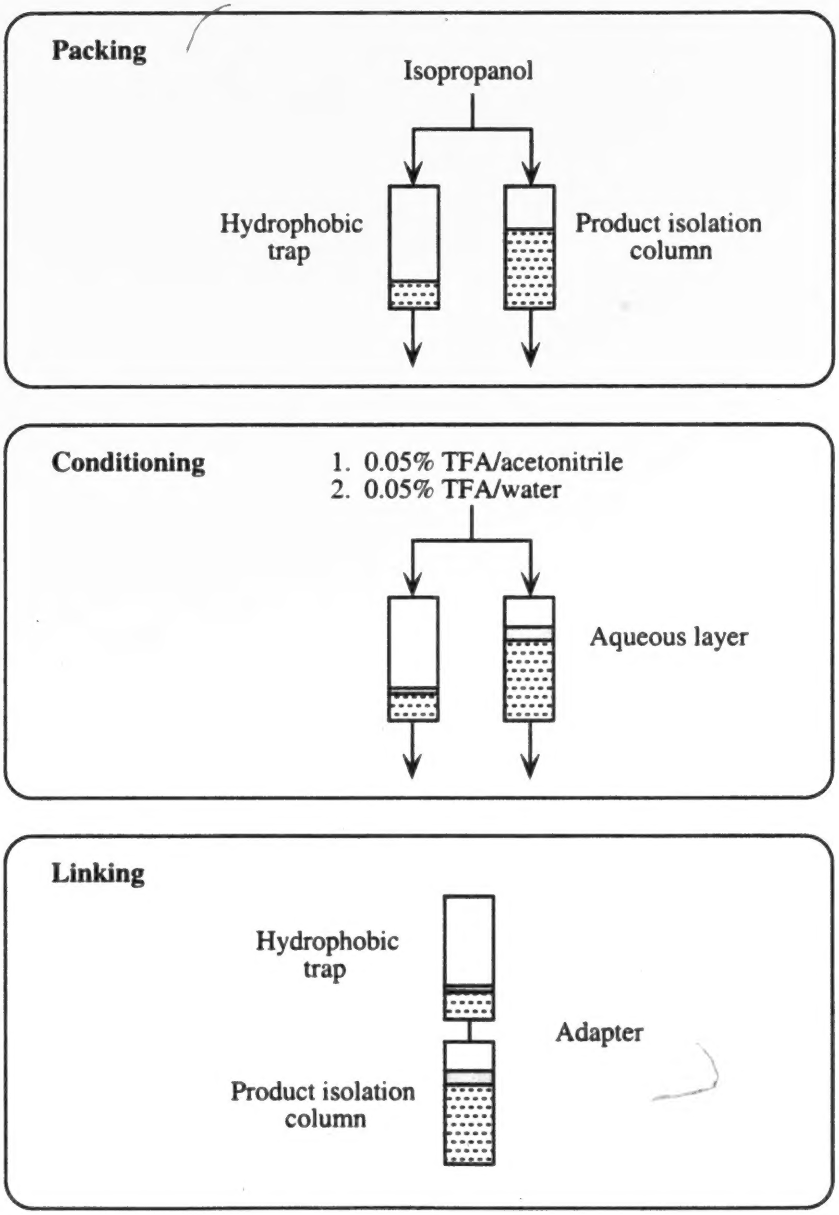

Packing
Isopropanol
Hydrophobic trap
Product isolation column
Conditioning
1. 0.05% TFA/acetonitrile
2. 0.05% TFA/water
Aqueous layer
Linking
Hydrophobic trap
Adapter
Product isolation column

Figure 57 Operation of Purification Unit

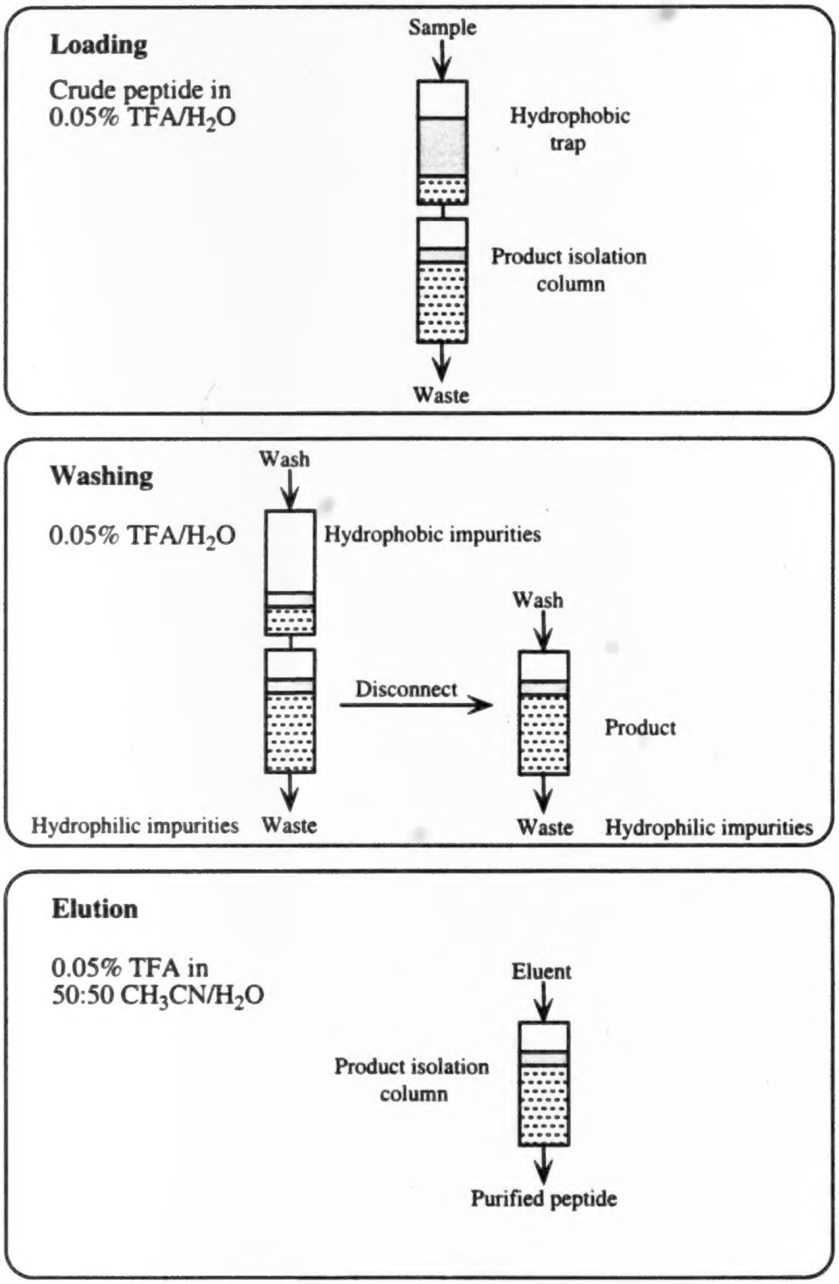

Loading
Crude peptide in 0.05% TFA/H_2O
Sample
Hydrophobic trap
Product isolation column
Waste
Washing
0.05% TFA/H_2O
Wash
Hydrophobic impurities
Wash
Disconnect
Product
Hydrophilic impurities
Waste
Waste
Hydrophilic impurities
Elution
0.05% TFA in 50:50 CH_3CN/H_2O
Eluent
Product isolation column
Purified peptide

Table 16 Purification Unit Packing and Operational Procedures

Packing Procedure

Insert bottom frit into SPE tube.

Add desired quantity of dry packing to SPE tube;
Wash down with isopropanol to form a bed.

Insert top frit to restrain packing bed.

Wet packing with one V_{tube} of isopropanol;
Drain under minimal vacuum, retaining 1 mm liquid above top frit to avoid draining bed.

Manually compress bed to remove air pockets and distribute packing evenly.

Operational Procedures

Conditioning

1 Vt_{ube} of CH_3CN to establish solvent layer.
1 V_{trap} or 1 V_{col} of 0.05% TFA/H_2O to equilibrate.

Retain 1 mm liquid above trap packing bed;
Retain 350 μL liquid above product isolation bed.

Loading

Sample dissolved in 0.05% TFA/H_2O

Dropwise flow (approximately 0.7 mL/min);
Retain liquid 1 mm above packing bed.

Washing

0.05% TFA/H_2O

Minimal wash of trap into column.
Disconnect trap from product isolation column.

Optimal wash of product isolation column;
Retain 1 mm liquid above packing.

Elution

50% CH_3CN / 0.05% TFA / H_2O

Minimal volume elution of product isolation column.
Drain column completely.

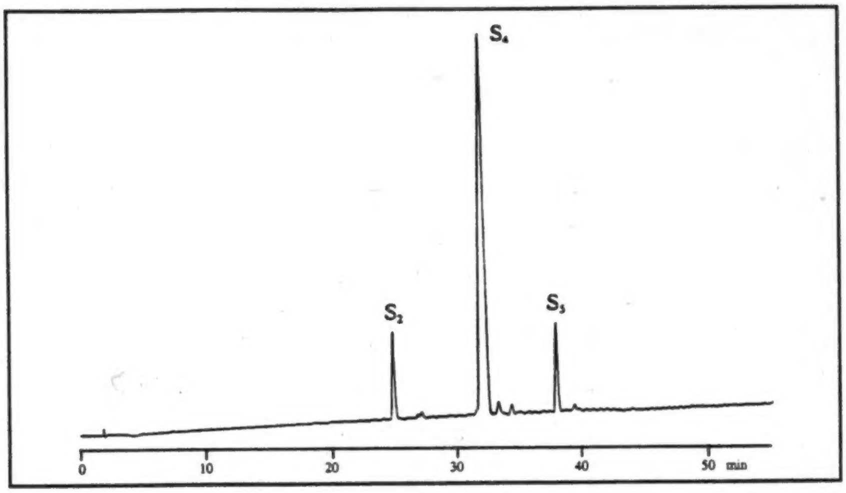
Table 17 Sequences of Synthetic Peptide Standards

Standard	Sequence
S_2	Ac- R G G G G L G L G K -amide
S_4	Ac- R G V G G L G L G K -amide
S_5	Ac- R G V V G L G L G K -amide

Ac = N^{α}-acetyl; amide = C^{α}-amide.
Variations in sequence are shown within the box.

Figure 58 **RP-HPLC of synthetic peptide standards S_2, S_4, and S_5 (1:9:1 sample mixture)**

Sample mixture: S_4 = product; S_2 = hydrophilic impurity; S_5 = hydrophobic impurity.



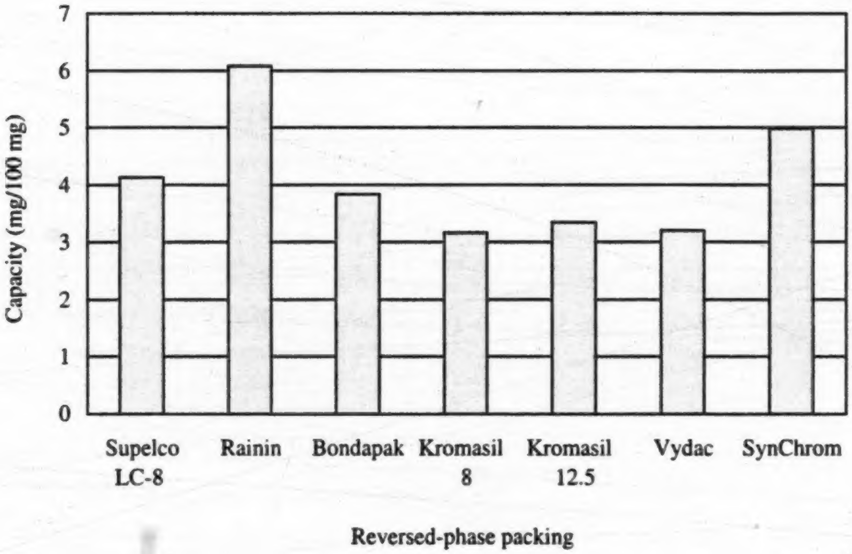
Column: Zorbax 300 SB C8 (150 x 4.6 mm ID)

Conditions: Linear AB gradient (0.5% B/min) at a flowrate of 1.0 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Table 18 Selected Silica-Based Reversed-Phase Packings

Packing	**Separation technique**	**Ligand**	**Particle Size (μm)**	**Pore Size (Å)**
Rainin	HPLC	C8	12	300
Bondapak	HPLC	C8	15-20	300
Kromasil-8	HPLC	C18	8	300
Kromasil-12.5	HPLC	C18	12.5	100
Vydac	HPLC	C18	20-30	300
SynChrom	HPLC	C18	30	300
Supelco LC-8	SPE	C8	40	300

Figure 59 Capacity Rating of Selected Reversed-Phase Packings



Packing capacity defined as mg peptide retained/100 mg packing using S_5 standard in 0.05% aqueous TFA.

Figure 60 **Variation of Packing Capacity with Particle and Pore Size**

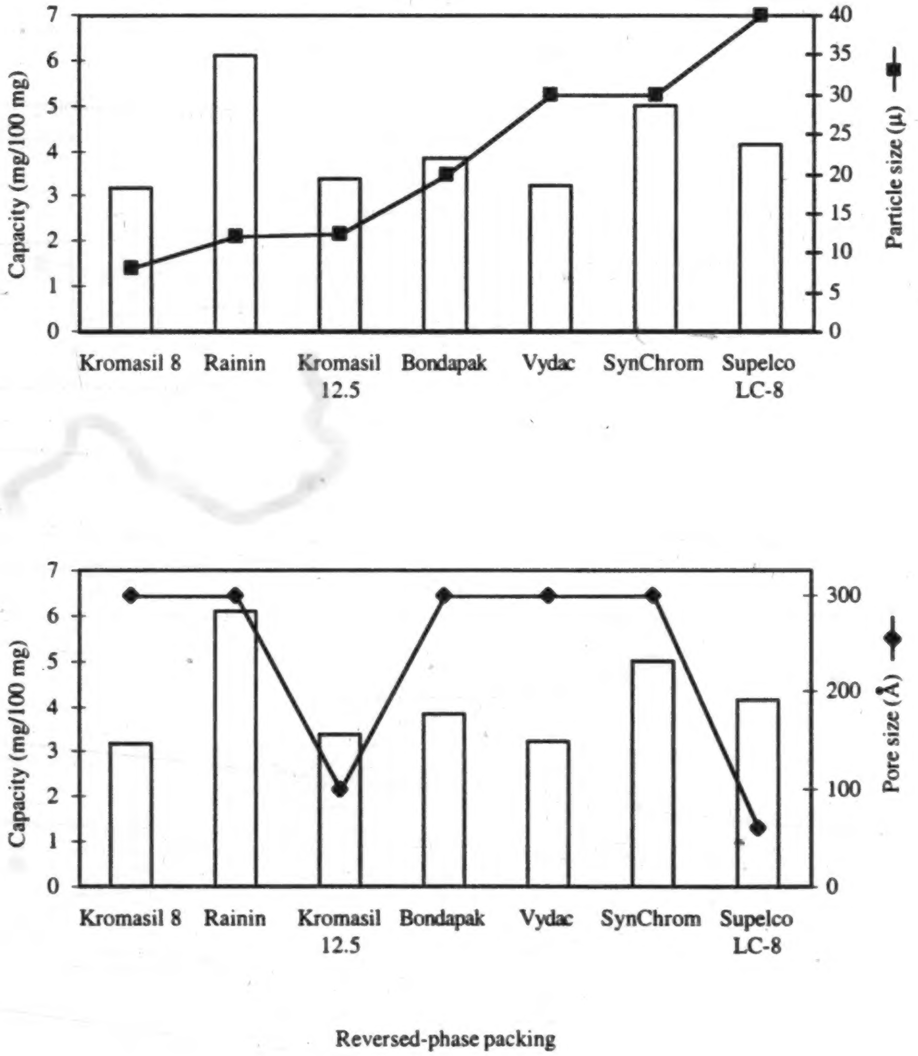
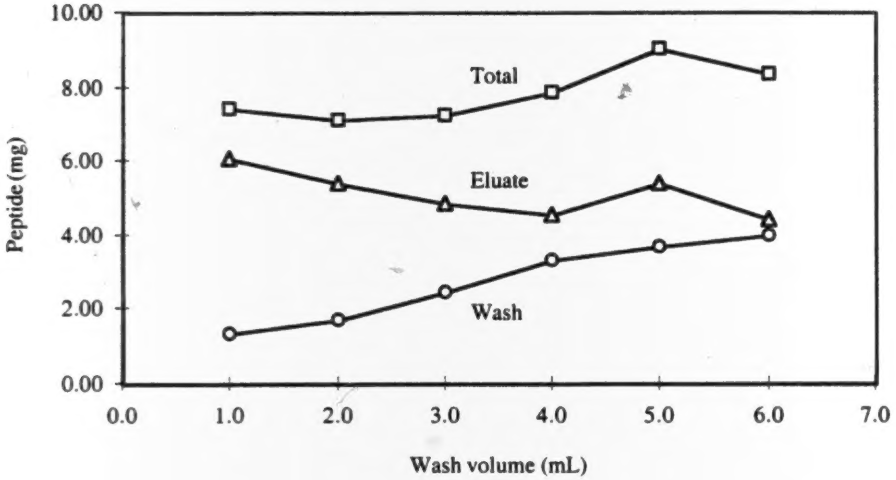


Figure 61 Effect of Wash Water on Peptide Retention by Packing



Column:	100 mg of Rainin packing
Load:	7 mg of peptide standard S_5
Wash:	0.05% aqueous TFA
Eluate:	50% acetonitrile / 0.05% aqueous TFA

Table 19 Initial Estimate of Trap-to-Column Packing Ratio

Performance objectives modified to account for absence of hydrophilic impurity S_2

Crude peptide sample	9:1	S_4 to S_5	Trap size set at 10 mg Rainin packing (i.e. minimum practical for assembly and operation) Peptide retention = 10 mg packing x 6.0 mg peptide/100 mg packing = 0.6 mg peptide
Hydrophobic trap	100% 5%	retention of S_5 loss of S_4	$100\%\ S_5 + 5\%\ S_4 = 0.6$ mg Sample load: $S_4 = 3.72$ mg $S_5 = 0.41$ mg $S_4 + S_5 = 4.13$ mg
Product isolation column	90%	retention of S_4	Estimated product isolation column size based on desired product retention Product retention = $90\%\ S_4 = 3.35$ mg Column size = 90% S4 /(6.0 mg peptide/100 mg packing) = 56 mg packing
			Trap:Column packing ratio estimate = 10 mg: 56 mg

Figure 62 **Effect of Trap Size on Trap Performance (Two Component Sample)**

Sample load = 4 mg.

S_4 to S_5 ratio = 9:1.

S_4 = product, S_5 = hydrophobic impurity.

Trap wash (into column) = 5 V_{trap} (100 µL for 10 mg packing
155 µL for 15 mg packing)

Retention of sample components on trap

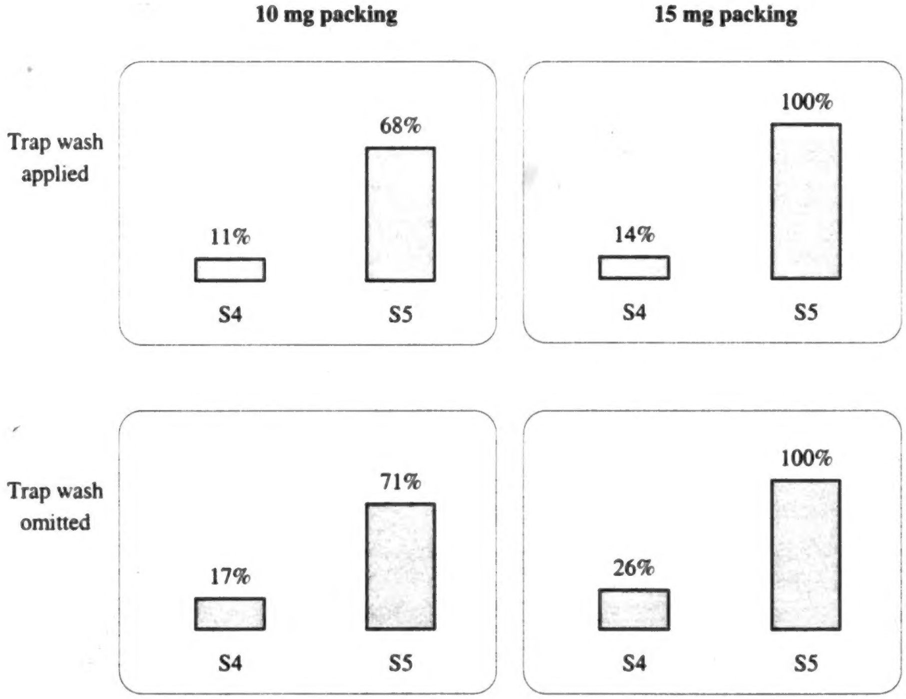


Figure 63 Purification Unit Performance with Optimized Trap (Two-Component Sample)

Sample load = 4 mg.

S_4 to S_5 ratio = 9:1.

S_4 = product; S_5 = hydrophobic impurity.

Trap wash (into column) = 5 V_{trap} (155 µL); column wash = 5 V_{col} (575 µL).

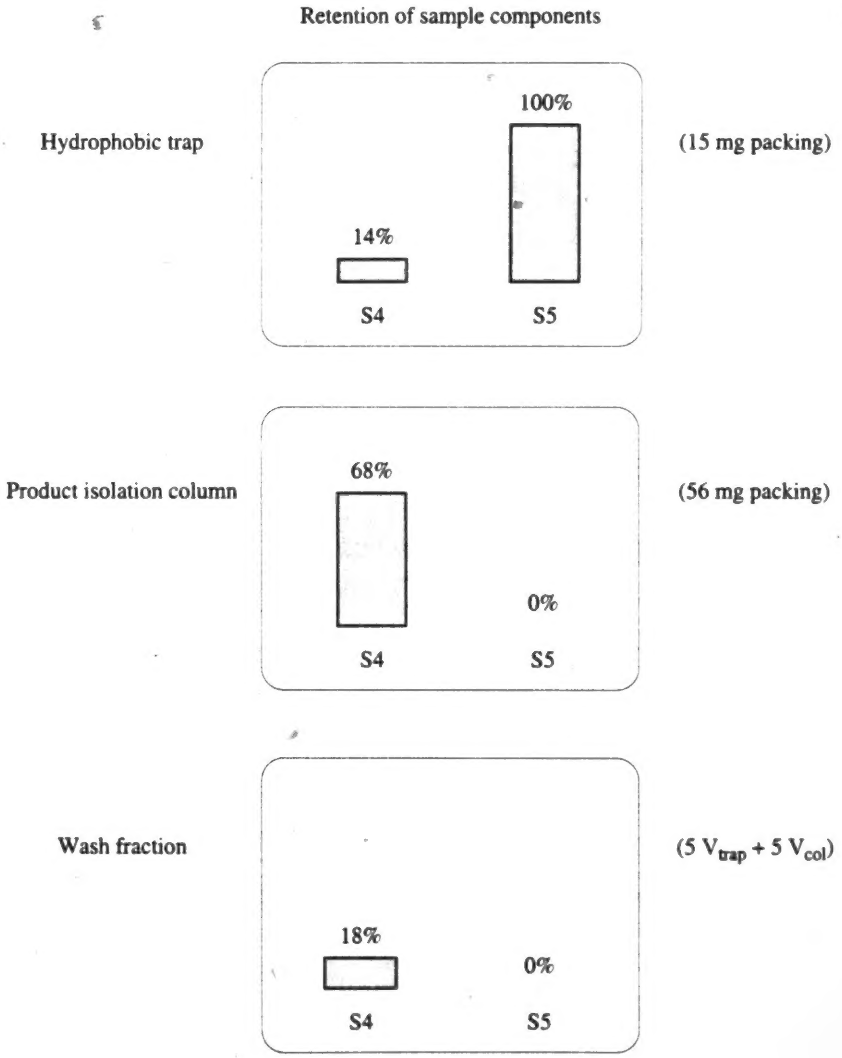


Figure 64 **Purification Unit Performance with Optimized Trap (Three Component Sample)**

Sample load = 4 mg.

S_2 to S_4 to S_5 ratio = 1:9:1.

S_2 = hydrophilic impurity; S_4 = product; S_5 = hydrophobic impurity.

Trap wash (into column) = 5 V_{trap} (155 μL); column wash = 7 V_{col} (805 μL).

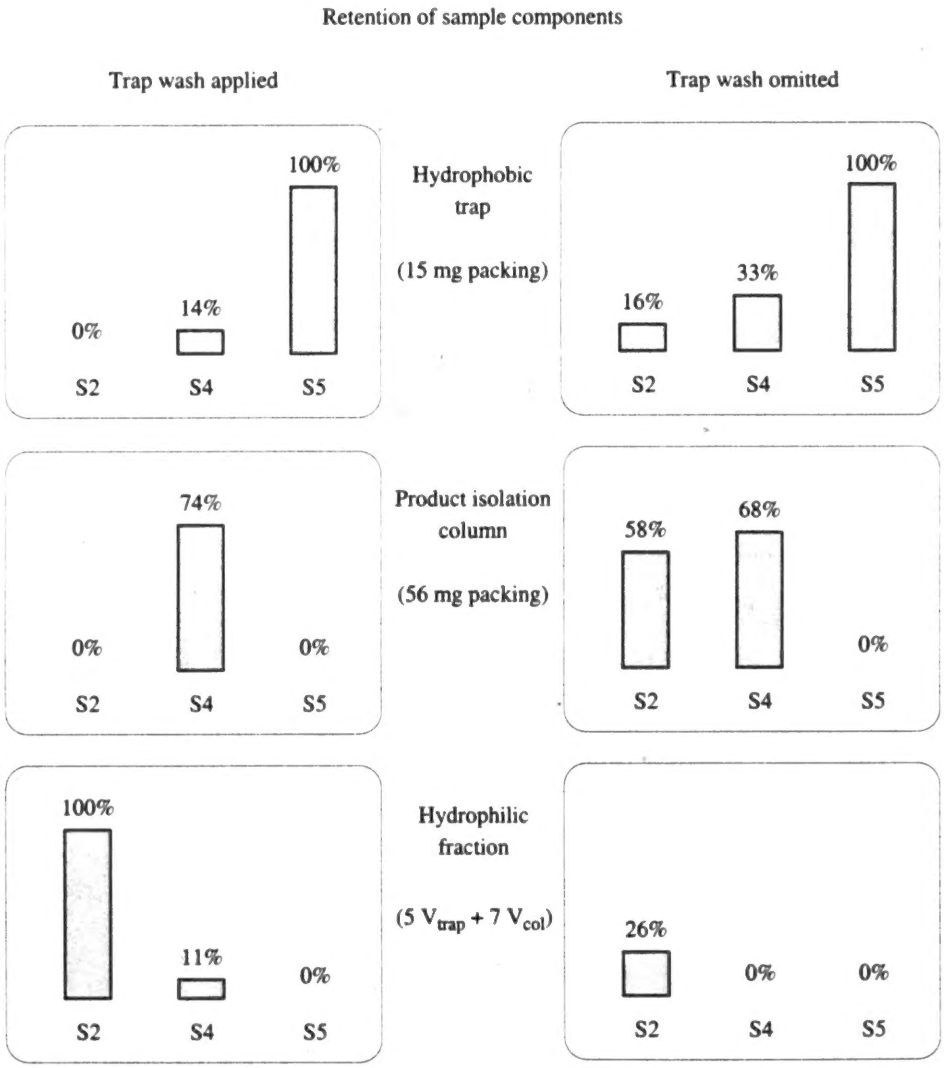


Figure 65 Effect of Product Isolation Column Size on Column Performance (Three Component Sample)

Sample load = 4 mg.

S_2 to S_4 to S_5 ratio = 1:9:1.

S_2 = hydrophilic impurity; S_4 = product; S_5 = hydrophobic impurity.

Trap = 15 mg packing; trap wash (into column) = 5 V_{trap} .

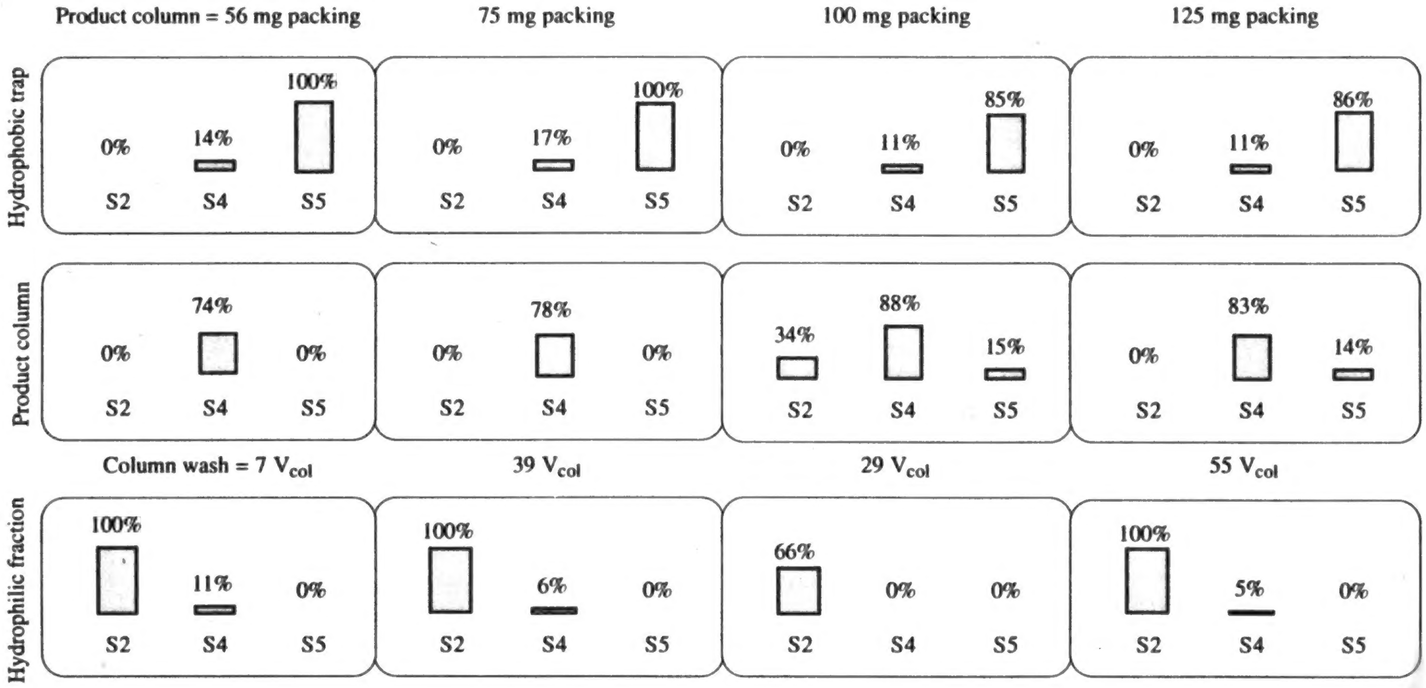


Figure 66 **Effect of Product Isolation Column Size on Sample Component Retention**

(Three Component Sample)

Sample load = 4 mg.
S_2 to S_4 to S_5 ratio = 1:9:1.
S_2 = hydrophilic impurity; S_4 = product; S_5 = hydrophobic impurity.
Trap = 15 mg packing; trap wash (into column) = 5 V_{trap} (150 μL).

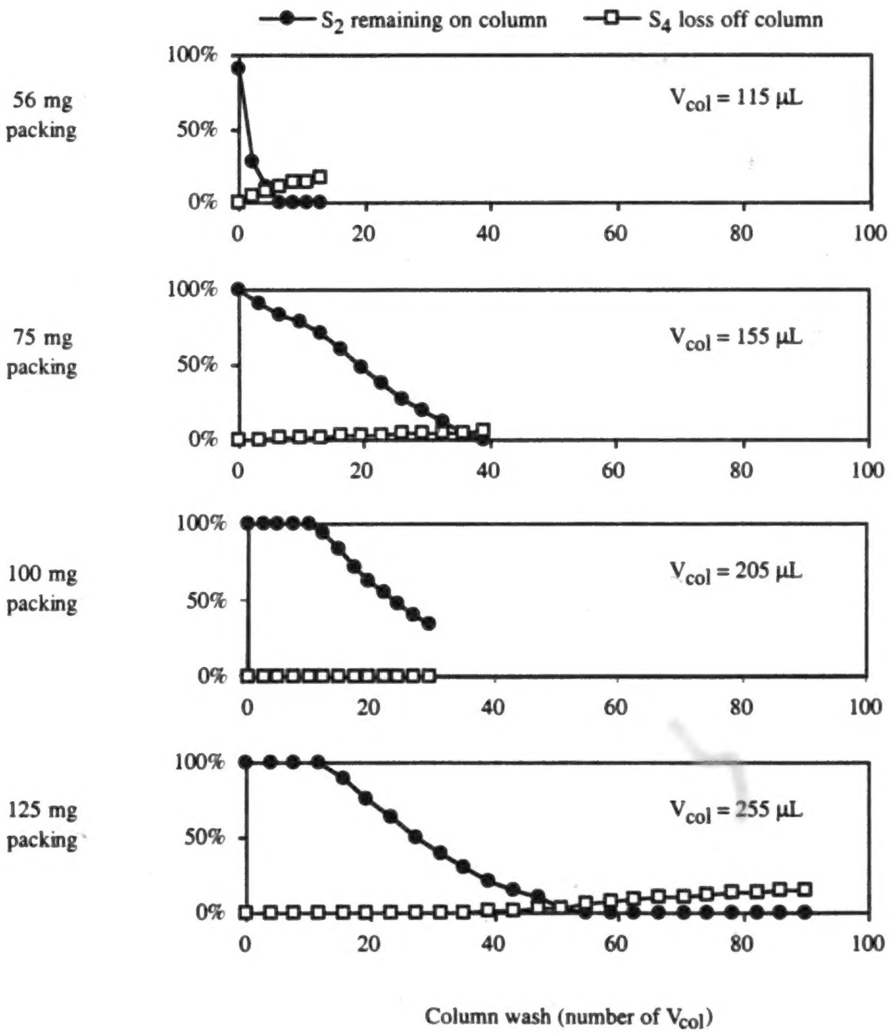


Figure 67 Purification Unit Performance at Different Sample Loads (Three Component Sample)

S_2 to S_4 to S_5 ratio = 1:9:1.

S_2 = hydrophilic impurity; S_4 = product; S_5 = hydrophobic impurity.

Trap-to-column packing ratio maintained at 15:100.

Trap wash (into column) = 5 V_{trap} (195 µL for 5 mg load; 390 µL for 10 mg load).

Column wash = 51 V_{col} (13 mL) for 5 mg load; 70 V_{col} (35 mL) for 10 mg load.

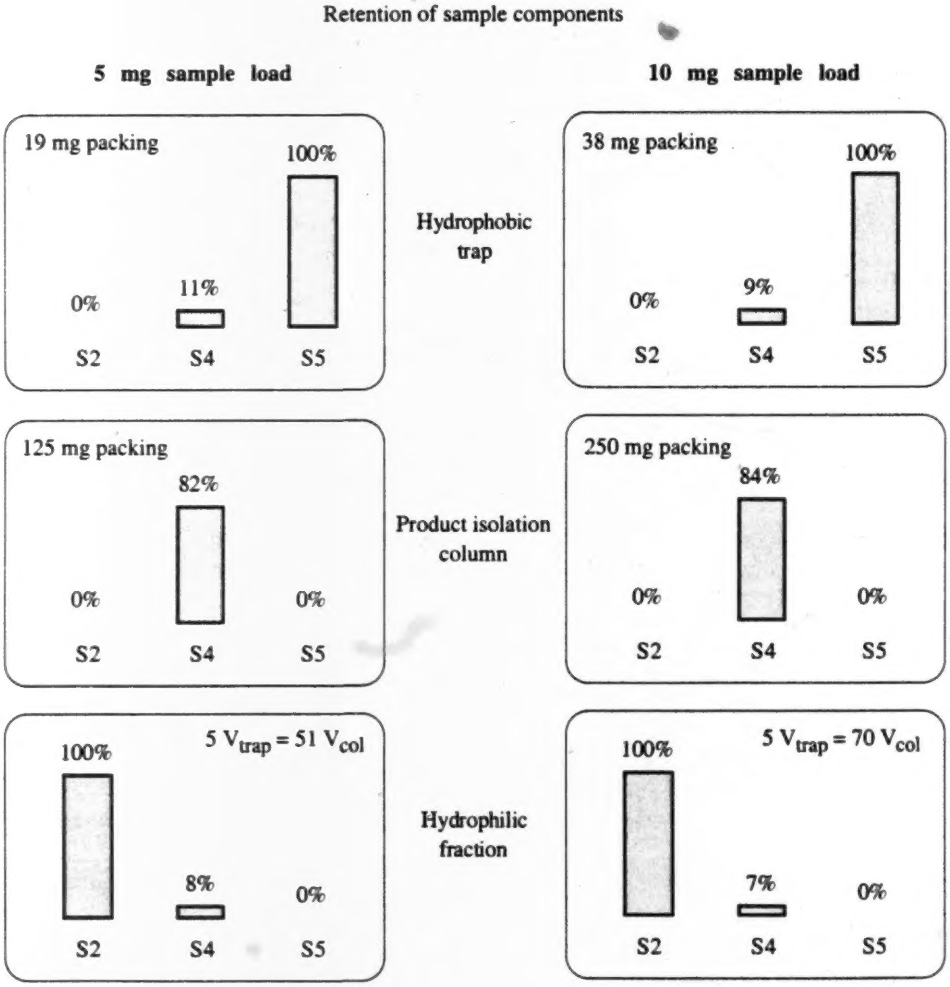


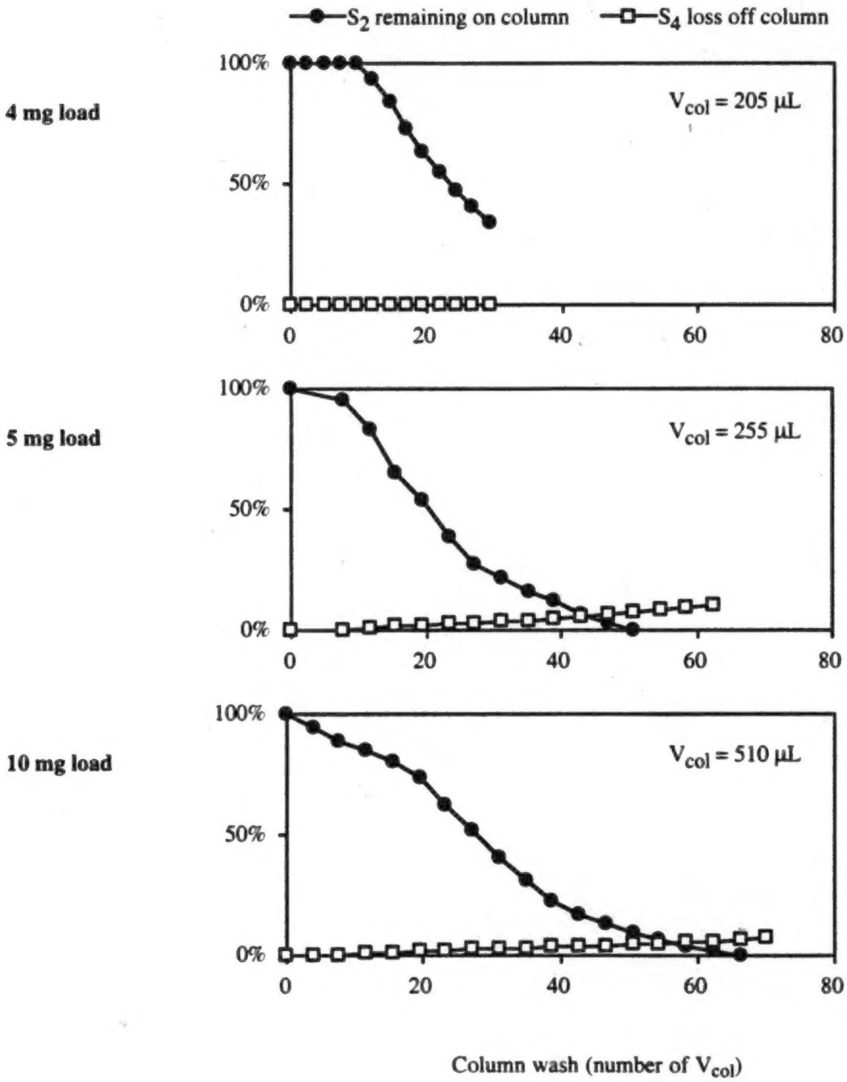
Figure 68 **Effect of Scale-up on Sample Component Retention (Three Component Sample)**

S_2 to S_4 to S_5 ratio = 1:9:1.

S_2 = hydrophilic impurity; S_4 = product; S_5 = hydrophobic impurity.

Trap-to-column packing ratio maintained at 15:100

Trap wash (into column) = 5 V_{trap} (195 µL for 5 mg load; 390 µL for 10 mg load).



7.3 Purification System Validation

As previously noted, the major challenge to multiple peptide purification is the variable nature of the synthesized crude peptides, characterized by a range of hydrophobicity, degree of purity, and scale of synthesis (i.e. sample load). The latter problem was addressed during the development of the purification system: the demonstrated ability to scale the purification unit to handle sample load on a linear basis, maintaining a constant trap-to-column packing ratio, permits the routine use of a protocol with standardized purification unit sizes accommodating different sample loads. The possibility of dealing in a similar manner with variation in crude purity, by establishing a few standardized trap-to-column packing ratios for expected ranges of sample impurities, was investigated during a more rigorous test of the system with a five-component mixture designed to represent the greater complexity of a typical crude peptide. The ability to separate product from multiple impurities over a range of hydrophobicities would validate the purification system design. The appropriate variation in hydrophobicity was provided by a second series of synthetic peptide standards, specifically constructed to span a greater hydrophobicity range than the S_2, S_4, and S_5 standards and to serve as a reference set for future protocols.

SDC Synthetic Peptide Standards

Three sets of synthetic peptide standards (GK, GI, and LI) were designed to cover a wide range of peptide hydrophobicity, and their sequences are presented in **Table 20**. Within each set there are 5 components (A_0-A_4) which are very closely separated in hydrophobicity, differing by the addition of 1 carbon atom with the substitution of an Ala residue for a Gly residue. A mixture of these components is used to represent a more complex crude peptide mixture produced by solid-phase peptide synthesis, in which a desired product (A_2) is contaminated by 2 hydrophilic impurities (A_0 and A_1) and 2 hydrophobic impurities (A_3 and A_4). Between sets, there is a difference in hydrophobicity range: the GK set incorporates Gly and Lys residues to provide the most hydrophilic set of standards; the GI set substitutes a much more hydrophobic residue, Ile, in place of the Lys residue; and the LI set incorporates two very hydrophobic residues, Leu and Ile, to provide the most hydrophobic set of standards. In addition, two Lys residues are present in all these standards to provide net positive charge and improve peptide solubility. These standards were all synthesized on the multiple peptide synthesizer using the standard protocol at 10-fold Fmoc-amino acid excess and a coupling time of 60 minutes. RP-HPLC chromatograms of the three sets are provided in **Figure 69**, demonstrating the three distinct hydrophobicity ranges; overlap exists only between component A_4 of the GI set and component A_0 of the LI set. A comparison of the three SDC sets with the (S_2, S_4, S_5) test set employed during system development reveals a correspondence with the middle set (GI), which falls within the hydrophobicity range of the test set.

Crude Complexity

Validation of the previously optimized purification system was conducted at a 5 mg sample load using a five-component mixture composed of the SDC GI set, maintaining the hydrophobicity range of the (S_2, S_4, S_5) standard set used during system development; the sample composition of 80% product, 10% hydrophilic impurity (5% each), and 10% hydrophobic impurity (5% each) was very close to the 1:9:1 ratio (corresponding to 82% product and 9% of each impurity) used with the (S_2, S_4, S_5) test set. At first glance, the performance of the 19:125 purification unit (trap and column sizes of 19 mg and 125 mg packing, respectively) appeared to reflect an inability to resolve the sample components due to their close hydrophobicities

(Figure 70). On closer inspection, the pattern of separation suggested the need for a larger hydrophobic trap: while adequate to retain all of the most hydrophobic impurity (A_4), the trap removed only 48% of the second, less hydrophobic impurity (A_3), resulting in extensive contamination of the product isolation column, and although product loss to the trap was expected, the presence of significant amounts of hydrophilic impurities A_0 and A_1 in the trap (2% and 11%, respectively) indicated that progressive sample displacement of one component by the next, more hydrophobic component was not allowed to reach completion. An almost 4-fold increase in trap size to 75 mg packing (approaching the size of the product isolation column) was required to ensure the complete retention of both hydrophobic impurities, as premature A_3 loss from the trap in the presence of product A_2 persisted with the use of smaller traps **(Figure 71)**. The substantial increase in size reflected the difficulty in resolving components A_2 and A_3. A similar increase in the trap wash (from 7 V_{trap} to 28 V_{trap}) was necessary to displace all hydrophilic impurities fron the hydrophobic trap. The elevated wash requirements to clear product from the much larger trap, along with a desire to simplify handling, prompted a change in operating procedure to apply wash to the entire purification unit (trap and column) rather than disconnecting the trap after a minimal wash; the wash volume was normalized as numbers of unit volumes (V_{unit}), based on the total solvated volume of the combined trap and column packing beds. The performance of the intact purification unit after re-optimization of trap size is presented in **Figure 72**. Using trap and column sizes of 75 mg and 125 mg packing respectively, the system was able to resolve five components exhibiting very minor differences in hydrophobicity to the extent of ensuring pure product in the product isolation column. Although the original performance objective of 80% product recovery could not be reached with the more complex mixture, complete removal of hydrophilic and hydrophobic impurities could be achieved by sacrificing sufficient product: 22% product loss in the hydrophobic trap and 13% product loss in the hydrophilic fraction, for a total pure product recovery of 65%.

Crude Purity (Sample Composition)

Purification unit performance depends on matching the crude purity to the trap-to-column ratio, as illustrated in **Figure 73**. Using the SDC GI standard set and maintaining a 5 mg sample load on the 75:125 purification unit, the sample composition was changed from 1:1:16:1:1 (i.e. 80% product and 5% hydrophilic and hydrophobic impurities each) to 1:1:6:1:1 (i.e. 60% product and 10% of each impurity); the purification unit wash was unchanged. In this case, the trap was sufficiently large to handle a 2-fold increase in the total hydrophobic impurity level without overflowing to the product isolation column, but the trap-to-column packing ratio was too large to recover pure product (insufficient product in the column prevented the complete displacement of both hydrophilic impurities). The purification unit was able to completely remove all hydrophobic impurities from the product as well as the most hydrophilic impurity, but 51% of the second hydrophilic impurity remained to contaminate the product. This problem could be overcome by continuing to wash the column separately (although this becomes more complicated and time consuming as the number of crude peptides to be purified rises). However, the reverse situation in which the trap-to-column packing ratio is too small for the level of impurities cannot be rescued: the product would be contaminated by both hydrophobic and hydrophilic impurities. These results emphasize the necessity of estimating the purity of the crude peptides (or more accurately, the purity of suspected poor synthesis products to determine the maximum trap required) by obtaining a few analytical RP-HPLC elution profiles; this would allow the future selection of an appropriate trap-to-column packing ratio from a limited number of standard ratios provided for routine use.

Peptide Hydrophobicity Range

A multiple peptide purification system must also be able to handle a range of crude peptide hydrophobicities, and this feature was investigated by applying a 5 mg sample load with a 1:1:16:1:1 component ratio (corresponding to 80% product and 5% of each hydrophilic and hydrophobic impurity) to the purification unit using each of the three SDC standard sets. The results (presented in **Figure 74**) suggest that these standards could be used as a reference set to adjust washing requirements for the hydrophobicity range of the crude peptide. The performance of the purification unit with the most hydrophobic standard set (LI) was lowered by incomplete removal of hydrophilic impurity A_1, although the 8% A_1 remaining represents a product contamination of only 0.7% (i.e. column retention 8%/68% x 5%/80% level in crude). This reflects the stronger interaction between peptide and packing due to the greater hydrophobicity of the SDC standard set. The advantage of this system is the ability to overcome this problem by increasing the wash volume used during the purification to displace the impurity from the column to the hydrophilic fraction; there may be potential to increase product recovery by continuing to direct the wash through the complete purification unit (trap and column) since considerable product (32%) remained in the trap. The performance achieved with the most hydrophilic standard set (GK) shows the converse trend: slightly incomplete removal of the hydrophobic impurity A_3 from the product and a greater product loss to the hydrophilic fraction (although the overall product recovery is similar). The separation is still excellent for a considerable change in hydrophobicity: only 0.7% of the A_3 impurity escaped the trap to contaminate the product, representing a product contamination of less than 0.1% (i.e. column retention 0.7%/68% x 5%/80% level in crude). This problem could also be overcome by adjusting the wash water: a decrease could prevent the displacement of the hydrophobic impurity from the trap and potentially reduce product loss to the hydrophilic fraction. The GK standard set may also point to the limit of the resolving power of this technique: the apparent premature loss of hydrophobic impurity A_3 from the trap in the presence of product A_2 suggests that the close hydrophobicity of the two standards (the closest of any of the standards) may be on the borderline of what can be separated.

These results extend the boundaries of the purification system beyond scaling for sample load on the basis of constant trap-to-column packing ratio in a two-stage purification unit. Variable crude purity can be accommodated by adjusting the ratio for the poorest quality product of the multiple peptide synthesis (i.e. the maximum ratio required) which will also achieve successful separation of higher quality crudes; peptide hydrophobicity (as predicted using hydrophobicity coefficients such as provided by Guo *et al.*, 1986) can be accommodated by comparison with a reference set of SDC standards to select an appropriate protocol with adjusted washing requirements best suited to the peptide. Standard protocols employing a limited number of standardized trap-to-column packing ratios and wash volumes can be envisaged, capable of handling the majority of crude peptides produced on the multiple peptide synthesizer.

Table 20 Sequences of SDC Synthetic Peptide Standard Sets

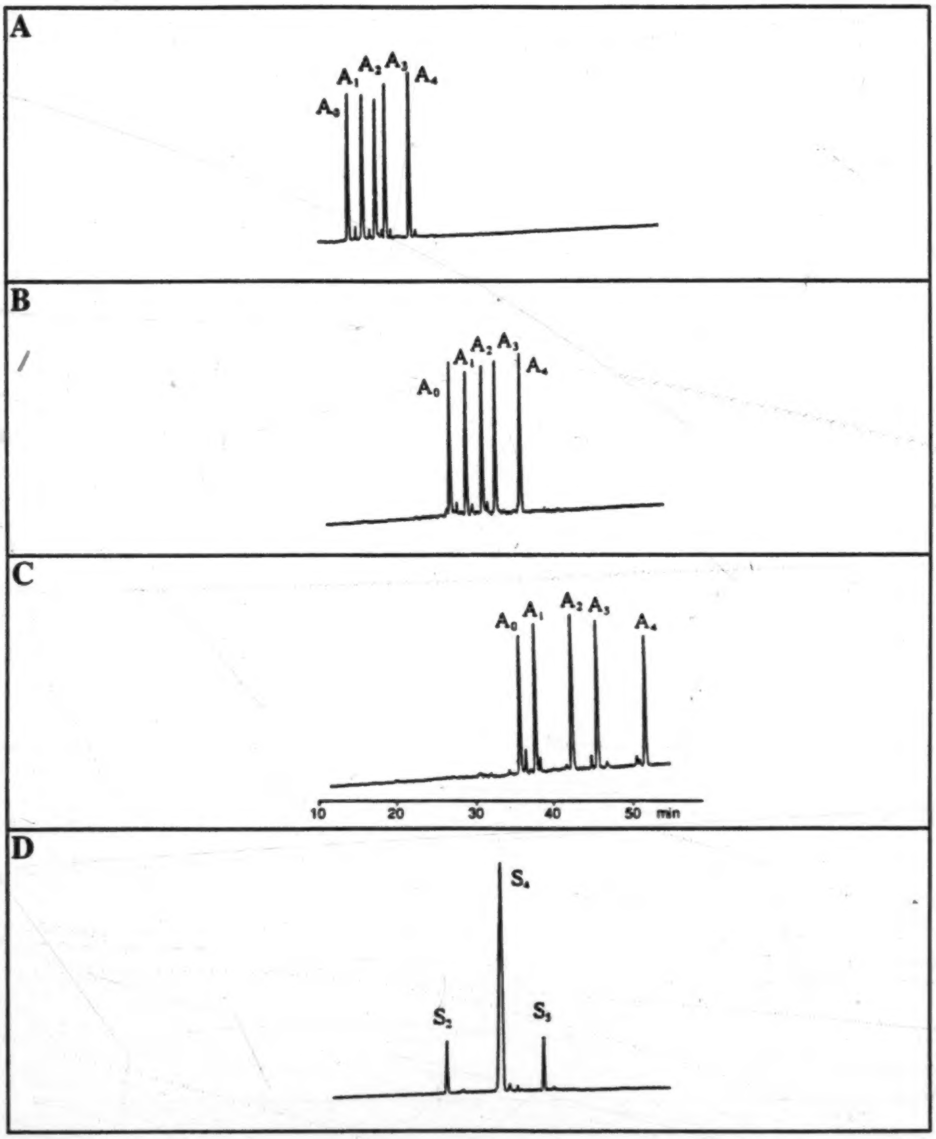
SDC Set	Standard	Sequence
GK	A_0	Ac- K G V G G K G G V G K -amide
	A_1	K G V G G K A G V G K
	A_2	K G V G A K A G V G K
	A_3	K G V G A K A A V G K
	A_4	K G V A A K A A V G K
GI	A_0	Ac- K G V G G I G G V G K -amide
	A_1	K G V G G I A G V G K
	A_2	K G V G A I A G V G K
	A_3	K G V G A I A A V G K
	A_4	K G V A A I A A V G K
LI	A_0	Ac- K L V G G I G G V G K -amide
	A_1	K L V G G I A G V G K
	A_2	K L V G A I A G V G K
	A_3	K L V G A I A A V G K
	A_4	K L V A A I A A V G K

Ac = N$^{\alpha}$-acetyl; amide = C$^{\alpha}$-amide.

Variations in sequence of the standards are shown within boxes.

Figure 69 RP-HPLC of synthetic peptide standards: Comparison of SDC sets with (S_2, S_4, S_5) test set

Panel A: GK set.
Panel B: GI set.
Panel C: LI set.
Panel D: (S_2, S_4, S_5) test set



Column: Zorbax 300 SB C8 (150 x 4.6 mm ID)
Conditions: Linear AB gradient (0.5% B/min) at a flowrate of 1.0 mL/min, Eluent A is 0.05% aqueous TFA and Eluent B is 0.05% TFA in acetonitrile

Figure 70 Performance of Purification Unit
(Five-Component Sample)

Sample load = 5 mg.
SDC GI set: A_o to A_1 to A_2 to A_3 to A_4 ratio = 1: 1 : 16 : 1 : 1 (i.e. 80 % product).
A_2 = product; A_3, A_4 = hydrophobic impurities; A_1, A_2 = hydrophilic impurities.
Trap wash (into column) = 7 V_{trap} (215 μL); column wash = 114 V_{col} (29 mL).

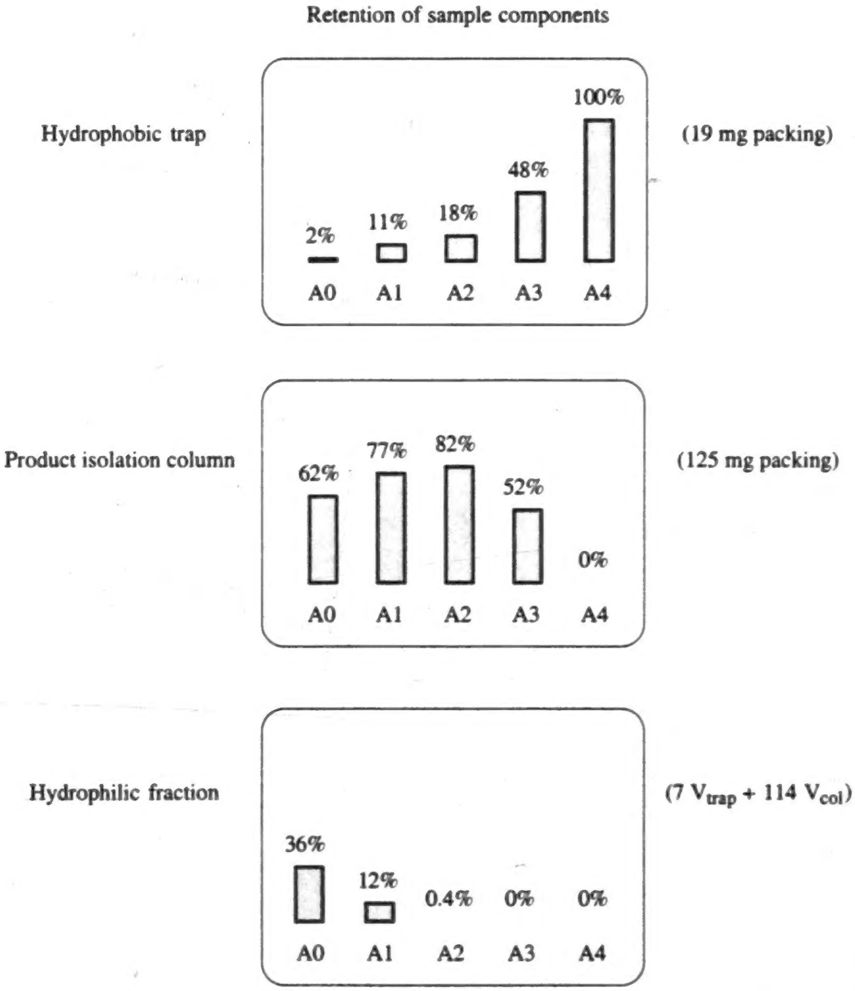


Figure 71 Effect of Hydrophobic Trap Size on Trap Performance (Five Component Sample)

Sample load = 5 mg.

SDC GI set: A_o to A_1 to A_2 to A_3 to A_4 ratio = 1: 1 : 16 : 1 : 1 (i.e. 80 % product).

A_2 = product; A_3, A_4 = hydrophobic impurities; A_1, A_2 = hydrophilic impurities.

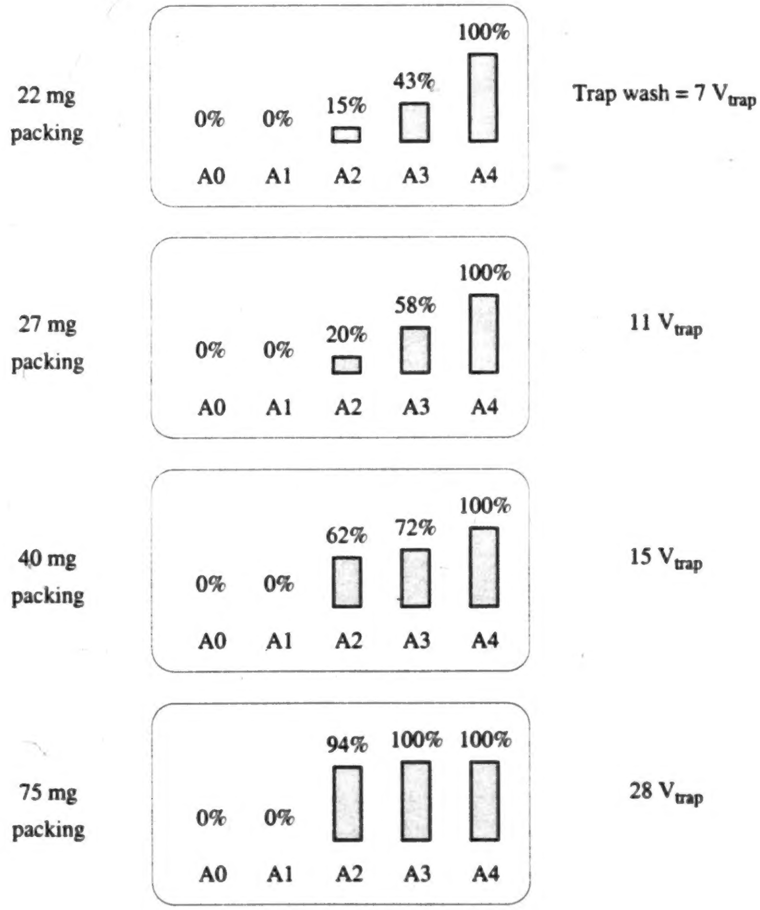


Figure 72 Performance of Re-optimized Purification Unit

(Five-Component Sample)

Sample load = 5 mg.

SDC GI set: A_o to A_1 to A_2 to A_3 to A_4 ratio = 1: 1 : 16 : 1 : 1 (80 % product).

A_2 = product; A_3, A_4 = hydrophobic impurities; A_1, A_2 = hydrophilic impurities.

Purification unit wash proceeds through trap and column.

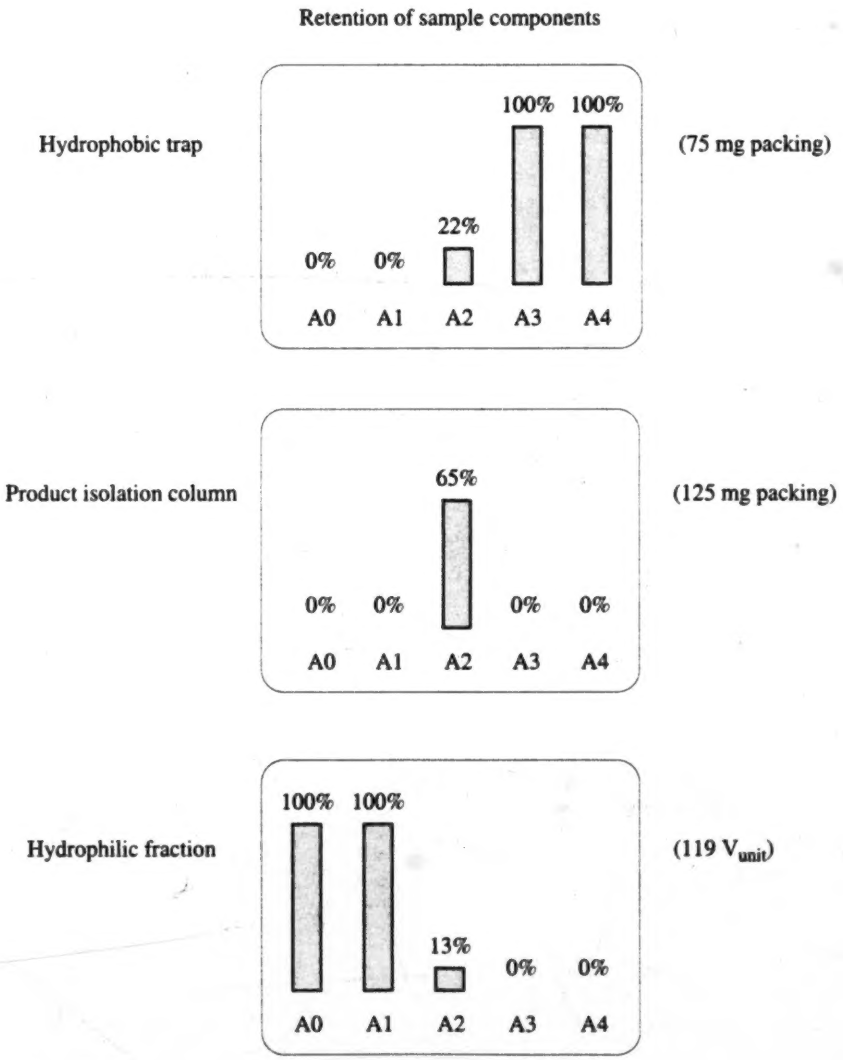


Figure 73 Effect of Sample Composition on Purification Unit Performance

(Five-Component Sample)

Sample load = 5 mg.

SDC GI set: A_0 to A_1 to A_2 to A_3 to A_4 ratio = 1: 1 : 6 : 1 : 1 (i.e. 60 % product).

A_2 = product; A_3, A_4 = hydrophobic impurities; A_1, A_2 = hydrophilic impurities.

Trap = 75 mg packing; column = 125 mg packing.

Purification unit wash = 119 V_{unit} (49 mL).

Retention of sample components

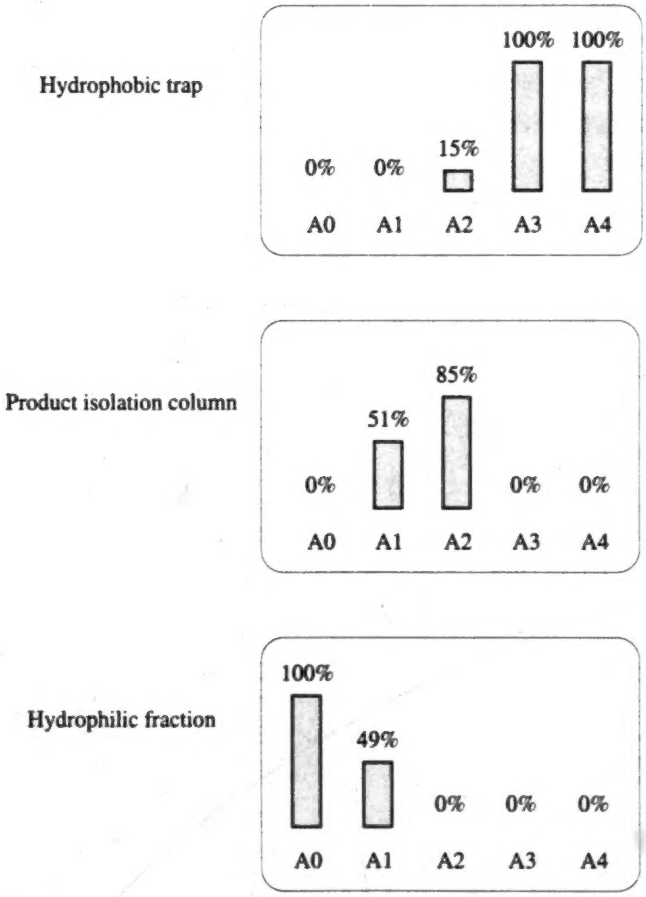


Figure 74 **Effect of Hydrophobicity Range on Purification Unit Performance (Comparison of SDC Standard Sets)**

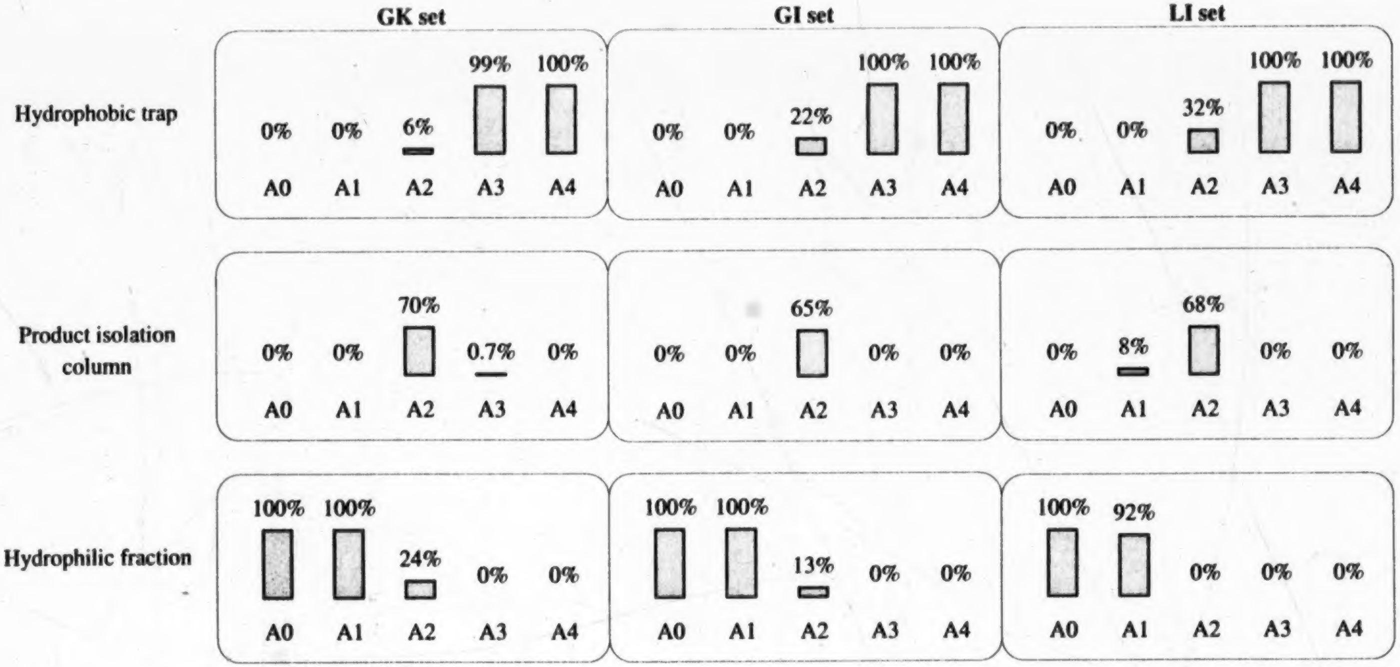
Sample load = 5 mg.

A_2 = product; A_3, A_4 = hydrophobic impurities; A_1, A_2 = hydrophilic impurities.

SDC GI set: A_0 to A_1 to A_2 to A_3 to A_4 ratio = 1: 1 : 16 : 1 : 1 (i.e. 80% product).

Trap = 15 mg; column = 125 mg.

Purification unit wash = 119 V_{unit} .



Chapter 8 CONCLUSIONS AND RECOMMENDATIONS

The expanding utility of synthetic peptides in a wide range of biological and medical applications emphasizes the importance of bringing multiple peptide synthesis to general laboratory use. The application of multi-tasking robotics to peptide synthesis has resulted in the recent introduction of a much more elaborate programmable multiple peptide synthesizer such as the 396 MBS offered by Advanced ChemTech, which is designed in a modular format very similar to the prototype with both synthesis and cleavage capability. The trend towards a completely automated synthesizer with accessory software to plan common syntheses (such as the generation of peptide fragments from a given protein sequence) is very costly - up to $100,000 Can., removing automated multiple peptide synthesis beyond the reach of individual laboratories. Yet this degree of sophisticated hardware and automation is not required in the laboratory - in fact, a minimalistic approach has been adopted in the construction of the Multiblock system by Lebl and Krchnák (1997) to facilitate multiple synthesis in syringes: this system consists of a block holder for 42 syringes equipped with an array of sealing stoppers and individual drainage lines attached to a vacuum manifold system. Simple design alternatives used by the synthesizer prototype designed in this project, such as the valveless module with sparging capability for mixing and the parallel dispensing station, achieve reliable results at an affordable price with a reasonable degree of automation, and the recent commercial availability of SPE processing stations with drying attachments (equipped with independently controlled gas flow to each sample tube) provides a ready-made solution to the unique evaporator employed in the preliminary stage of peptide recovery. The prototype has undergone successful testing of hardware and selected methodology and is currently in routine use in a peptide synthesis laboratory, and a second generation model has been designed with microprocessor-controlled operation of the dispensing station and reaction module pressurization/aspiration to provide further automation of tedious synthetic steps at a fraction of the cost imposed by robotics; the programmable features of this model are provided in **Appendix C**. New instrument features include the use of relatively inexpensive peristaltic pumps for solvent and reagent delivery (in place of the typical high pressure pneumatic system employed by conventional automated single peptide synthesizers with attendant containment problems) and the extension of the modular approach to interchangeable 20- and 100-reactor dispenser and reaction modules mounted in a common cabinet. Commercialization of this synthesizer is underway, and is expected to accomplish the goal of affordable multiple peptide synthesis (in the initial range of $15,000-$20,000 Can.) for individual laboratories.

Considerable progress has been made on the development of a simple, rapid and affordable multiple peptide purification system. The standard use of RP-HPLC requires a considerable investment in instrumentation, and even the introduction of ultrafast RP-HPLC is insufficient to handle large numbers of peptide purifications due to its serial nature. The development of a parallel low pressure multiple purification system using modular solid phase extraction (SPE) technology and sample displacement chromatography (SDC) methodology has provided a remarkably effective and inexpensive alternative to complement the multiple peptide synthesizer. Equipment costs are a fraction of traditional RP-HPLC: SPE processing stations with independent valves to accommodate unequal flowrates in purification units (preventing dryout of packing) are now available in the range of only $1000-$1500 Can.. The purification system has demonstrated excellent separation of product from both hydrophobic and hydrophilic impurities over a range of hydrophobicity, crude composition, and sample load for a model system of synthetic standards; it remains to be tested on an actual synthesis project such as a sizeable series of analogues, using a standard protocol based on the predicted hydrophobicity.

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APPENDIX A

MULTIPLE PEPTIDE SYNTHESIZER

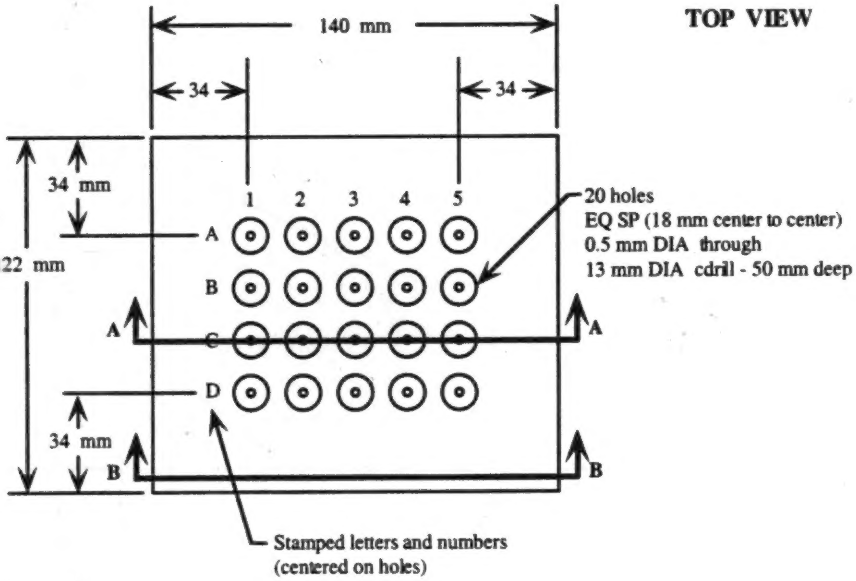
Parts & Materials
Technical Drawings

SYNTHESIZER PARTS & MATERIALS

- 1/8", 3/16" 316 stainless steel sheet
- Gas rotameter with regulating valve (0.1-1.0 L/min)
- Manual miniature teflon 3-valve distribution manifold
- Manual miniature on-off teflon valve
- Teflon tee connector
- Teflon tubing (0.5 mm ID, 1.5 mm OD)
- Tefzel unions, flangeless ferrules and nuts
- Stainless steel adjustable tension clamps
- Glass capillary tubing (0.5 mm ID, 6 mm OD)
- 316 stainless steel tubing (0.5 mm ID, 0.8 mm OD)
- C-flex tubing (1/8" ID, 1/4" OD)
- Polyethylene frits (13 mm dia, 20 μ nominal pore size)

20 REACTOR MODEL REACTOR BLOCK

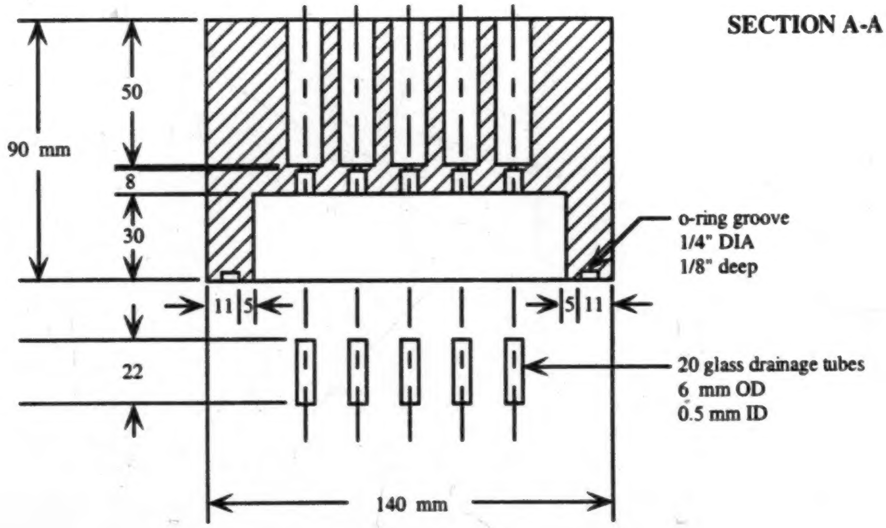
TOP VIEW


140 mm
34
34
34 mm
122 mm
34 mm
1
2
3
4
5
A
B
C
D
A
A
B
B

20 holes
EQ SP (18 mm center to center)
0.5 mm DIA through
13 mm DIA cdrill - 50 mm deep

Stamped letters and numbers
(centered on holes)

SECTION A-A

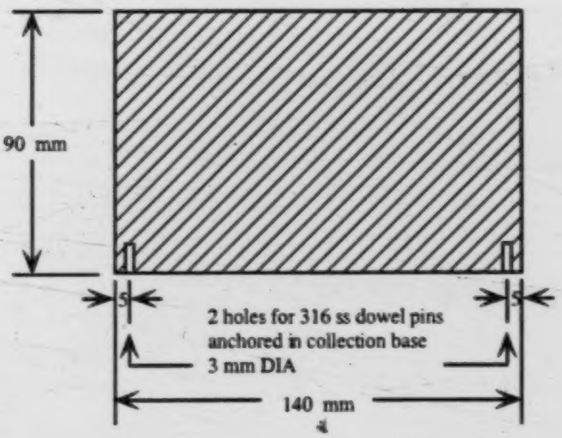

90 mm
50
8
30
11
5
5
11
22
140 mm

o-ring groove
1/4" DIA
1/8" deep

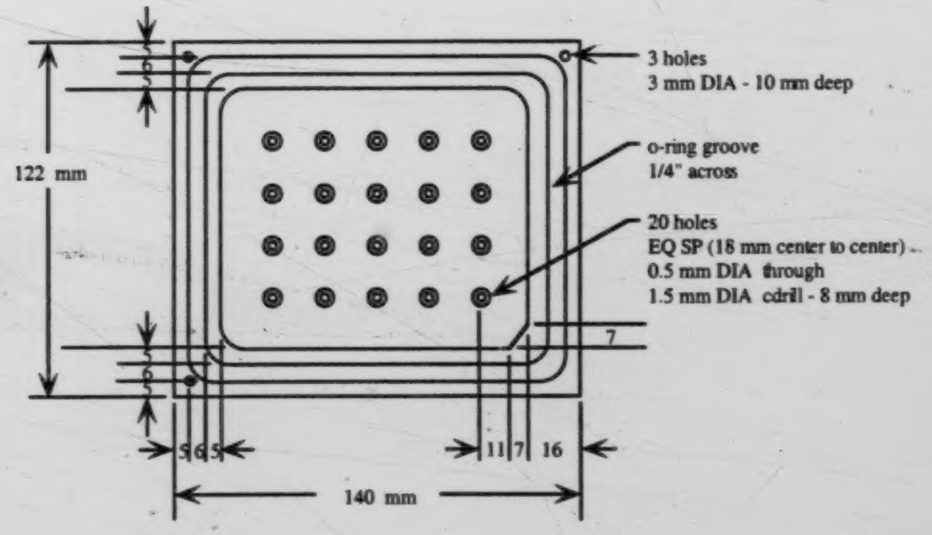
20 glass drainage tubes
6 mm OD
0.5 mm ID

20 REACTOR MODEL REACTOR BLOCK

SECTION B-B

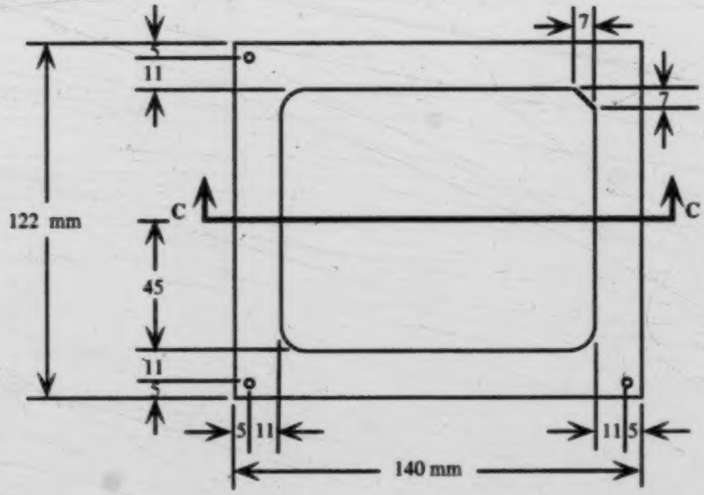

90 mm
5
5
2 holes for 316 ss dowel pins
anchored in collection base
3 mm DIA
140 mm

BOTTOM VIEW

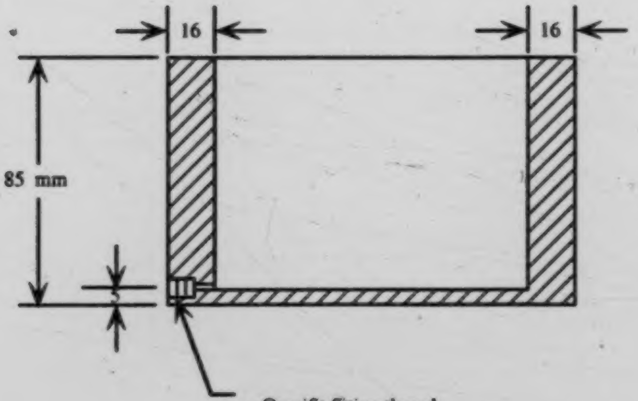

5
6
5
3 holes
3 mm DIA - 10 mm deep
o-ring groove
1/4" across
122 mm
20 holes
EQ SP (18 mm center to center)
0.5 mm DIA through
1.5 mm DIA cdrill - 8 mm deep
7
5
6
5
5 6 5
11 7 16
140 mm

20 REACTOR MODEL COLLECTION BASE

TOP VIEW


5
11
7
7
122 mm
C
C
45
11
5
5 11
11 5
140 mm

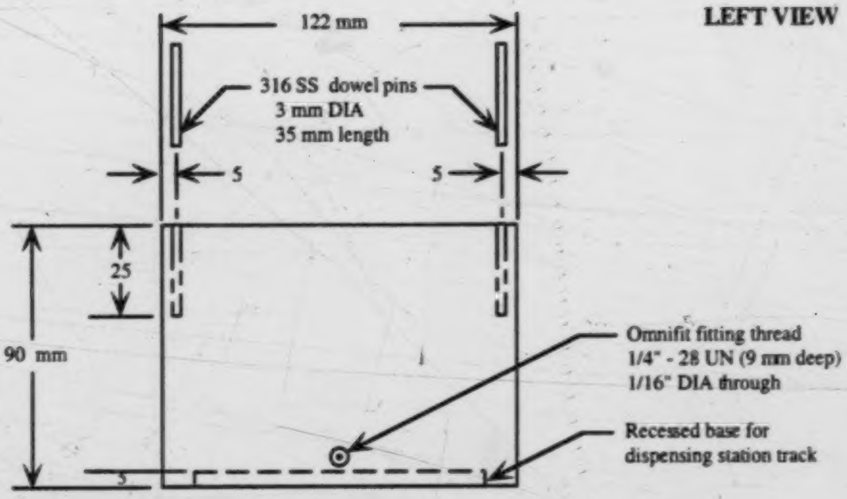
SECTION C-C


16
16
85 mm
5
5

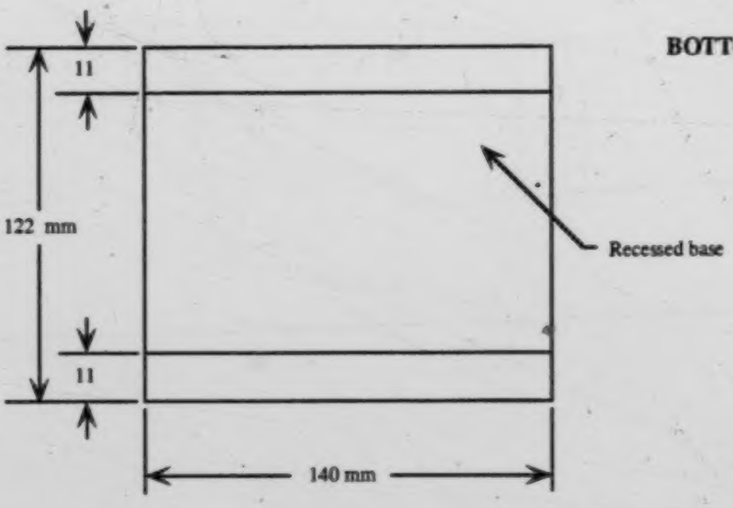
Omnifit fitting thread
1/4" - 28 UN (9 mm deep)
1/16" DIA through

20 REACTOR MODEL COLLECTION BASE

LEFT VIEW

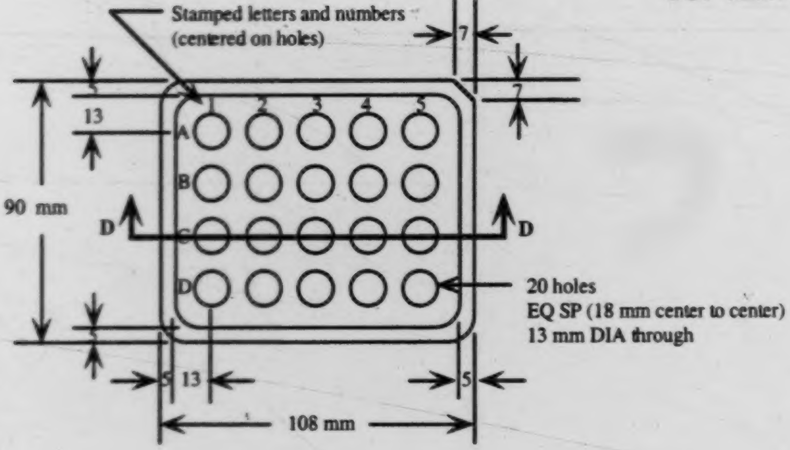

122 mm
316 SS dowel pins
3 mm DIA
35 mm length
5
5
25
90 mm
5
Omnifit fitting thread
1/4" - 28 UN (9 mm deep)
1/16" DIA through
Recessed base for
dispensing station track

BOTTOM VIEW

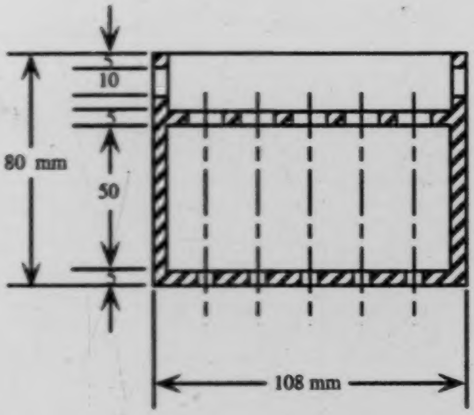

11
122 mm
11
140 mm
Recessed base

20 REACTOR MODEL COLLECTION TUBE RACK

TOP VIEW

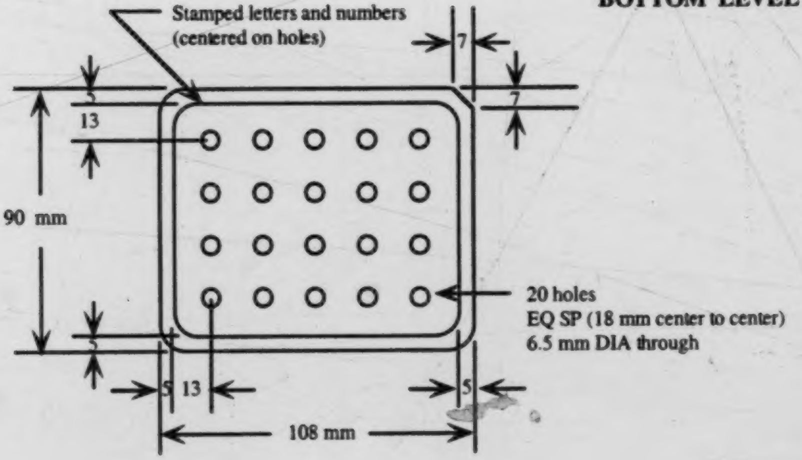

Stamped letters and numbers
(centered on holes)
7
7
5
13
1
2
3
4
5
A
B
C
D
90 mm
D
D
20 holes
EQ SP (18 mm center to center)
13 mm DIA through
5
5
13
5
108 mm

SECTION D-D

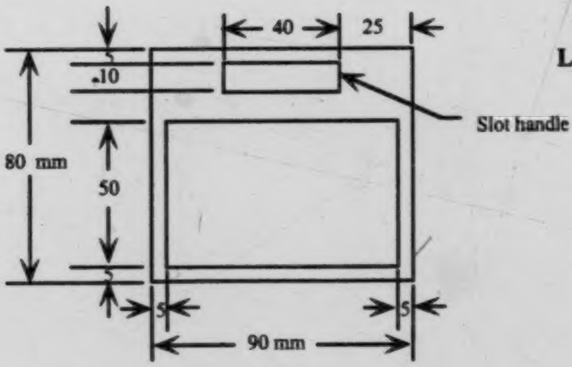

5
10
5
5
80 mm
50
5
108 mm

20 REACTOR MODEL COLLECTION TUBE RACK

BOTTOM LEVEL


Stamped letters and numbers
(centered on holes)
7
7
5
13
90 mm
5
20 holes
EQ SP (18 mm center to center)
6.5 mm DIA through
5
13
5
108 mm

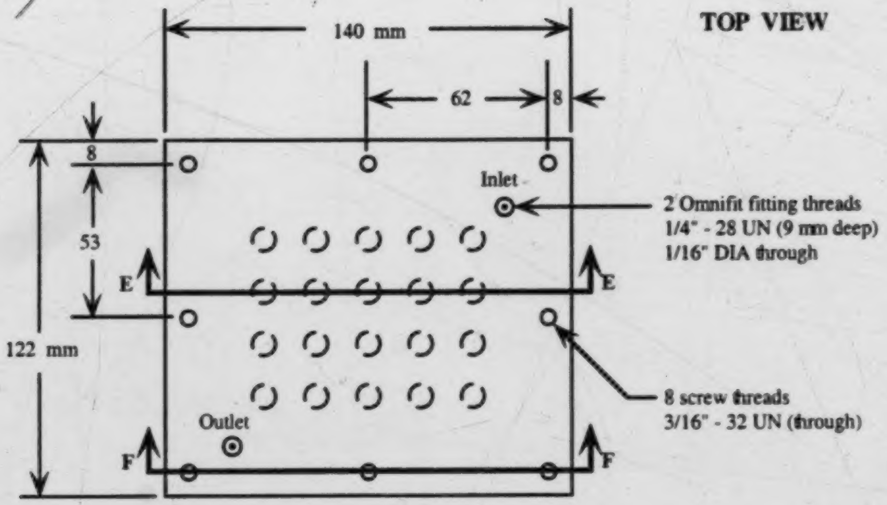
LEFT SIDE VIEW


40
25
5
10
Slot handle
80 mm
50
5
5
5
90 mm

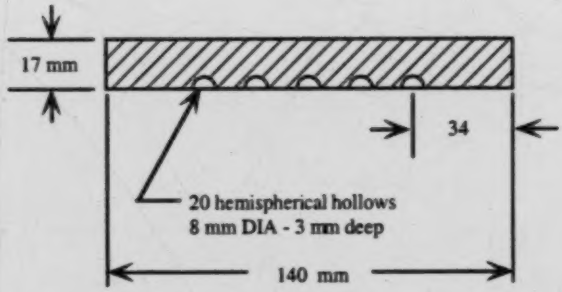
20 REACTOR MODEL

DISPENSER UPPER SECTION

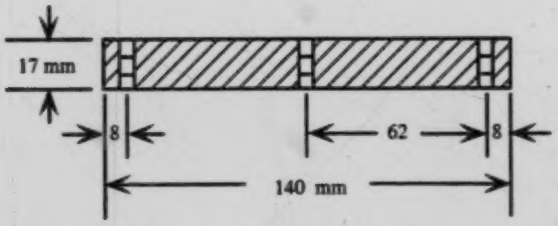
TOP VIEW


140 mm
62
8
8
53
122 mm
Inlet
E
E
Outlet
F
F
2 Omnifit fitting threads
1/4" - 28 UN (9 mm deep)
1/16" DIA through
8 screw threads
3/16" - 32 UN (through)

SECTION E-E

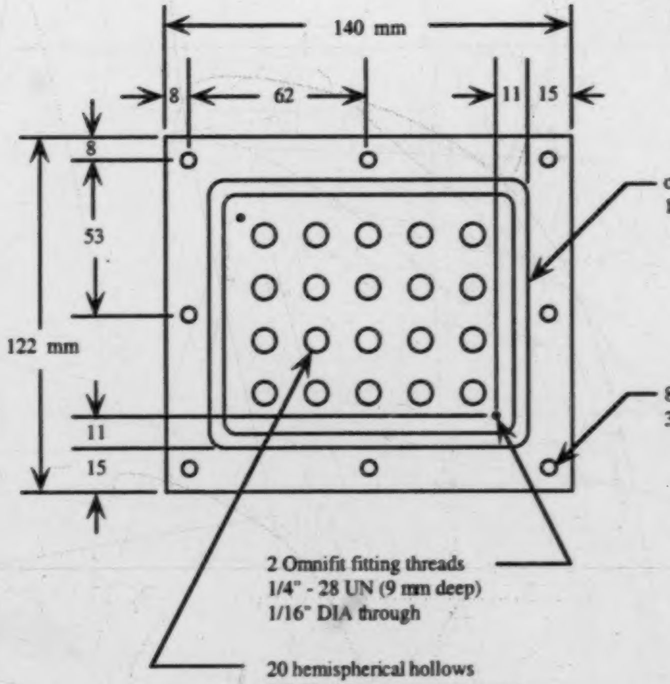

17 mm
34
20 hemispherical hollows
8 mm DIA - 3 mm deep
140 mm

SECTION F-F


17 mm
8
62
8
140 mm

20 REACTOR MODEL DISPENSER UPPER SECTION

BOTTOM VIEW


140 mm
8
62
11
15
8
53
122 mm
11
15

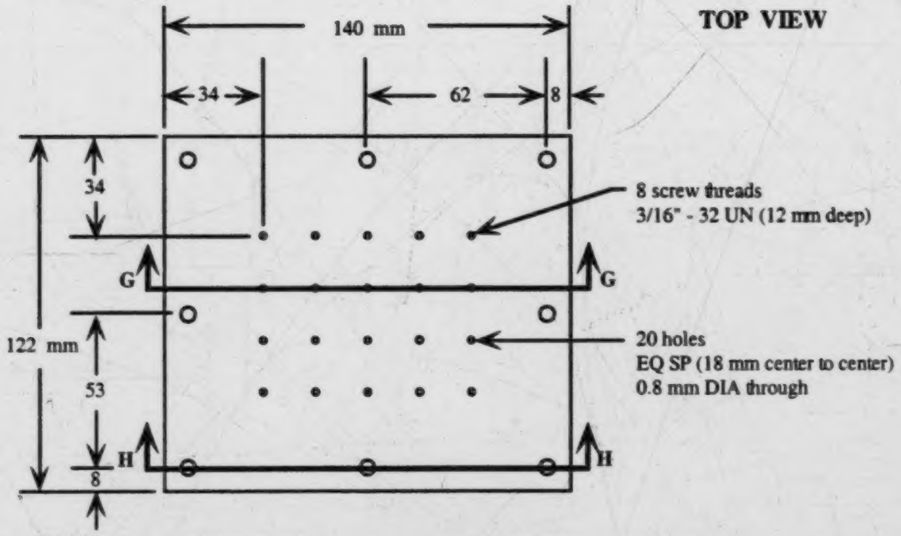
o-ring groove
1/4" across

8 screw threads
3/16" - 32 UN (through)

2 Omnifit fitting threads
1/4" - 28 UN (9 mm deep)
1/16" DIA through

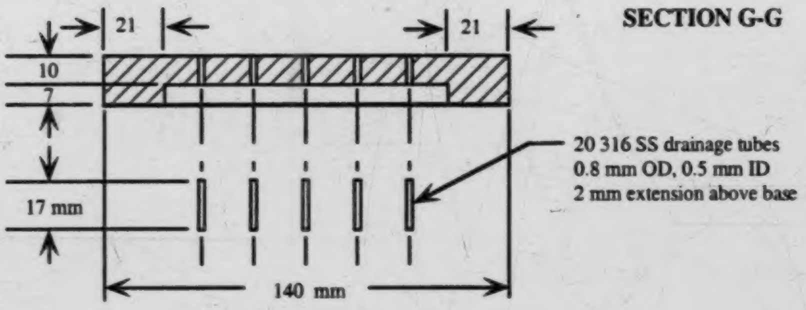
20 hemispherical hollows
EQ SP (18 mm center to center)
8 mm DIA - 3 mm deep

20 REACTOR MODEL DISPENSER BASE



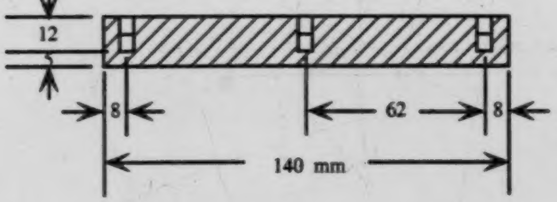
TOP VIEW

140 mm
34
62
8
34
8 screw threads
3/16" - 32 UN (12 mm deep)
G
G
122 mm
20 holes
EQ SP (18 mm center to center)
0.8 mm DIA through
53
H
H
8



SECTION G-G

21
21
10
7
20 316 SS drainage tubes
0.8 mm OD, 0.5 mm ID
2 mm extension above base
17 mm
140 mm

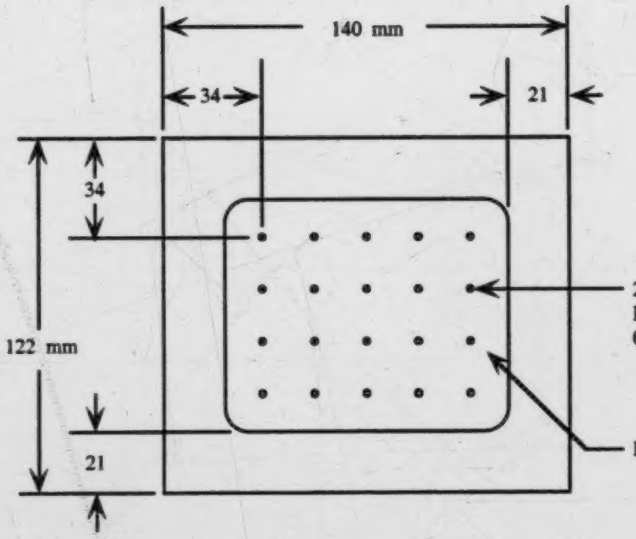


SECTION H-H

12
5
8
62
8
140 mm

20 REACTOR MODEL DISPENSER BASE

BOTTOM VIEW

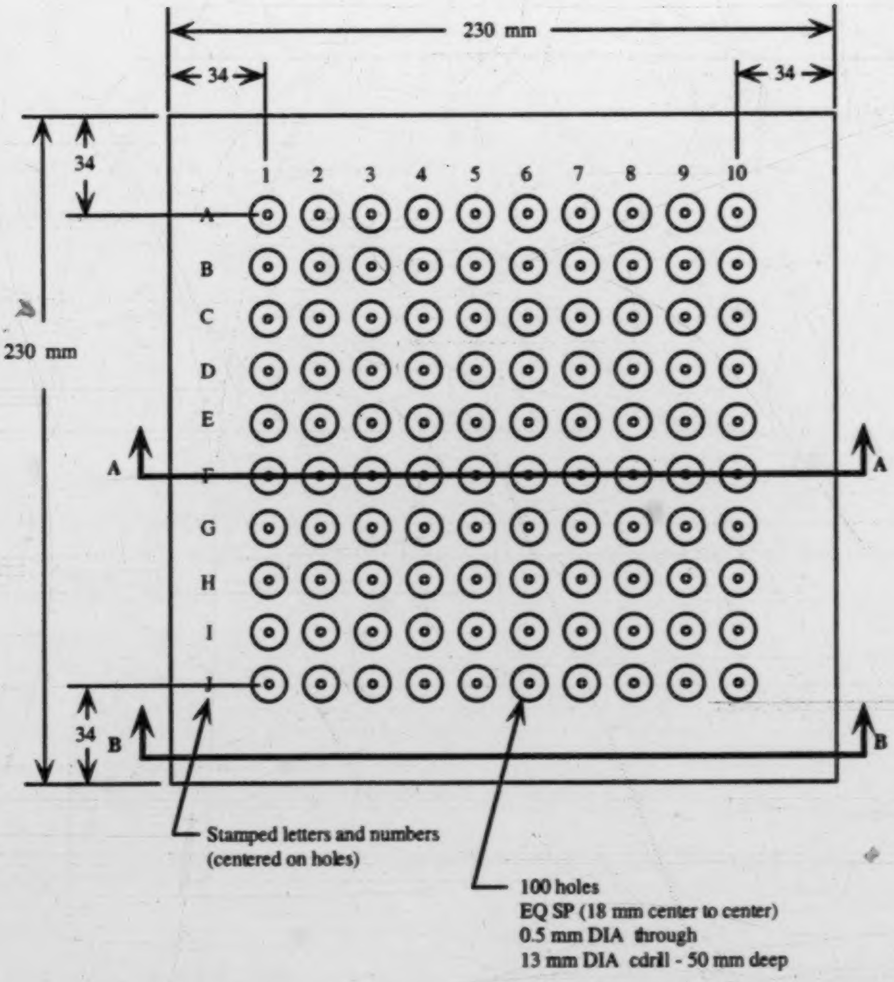

140 mm
34
21
34
122 mm
21

20 holes
EQ SP (18 mm center to center)
0.8 mm DIA through

Recessed base

100 REACTOR MODEL REACTOR BLOCK

TOP VIEW


230 mm
34
34
34
230 mm
34
1
2
3
4
5
6
7
8
9
10
A
B
C
D
E
F
G
H
I
J
A
A
B
B

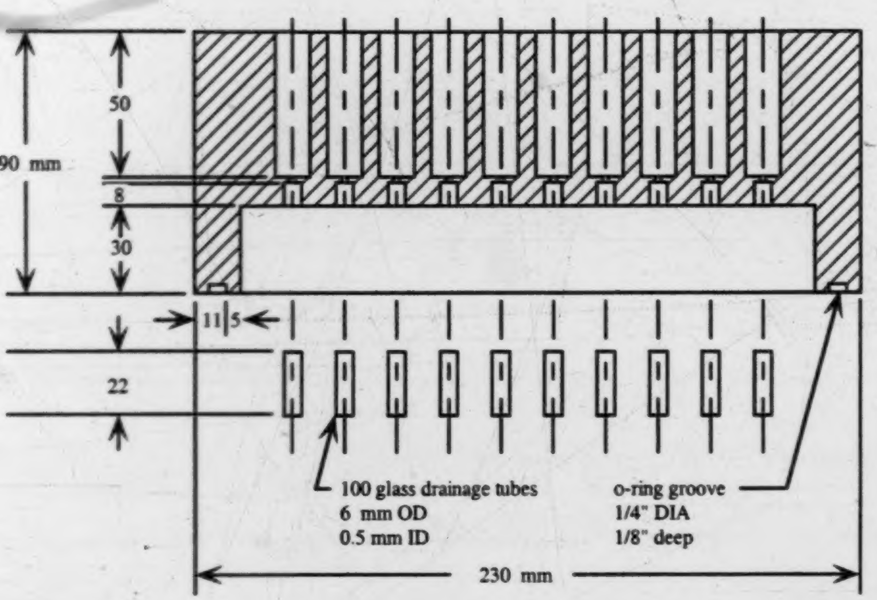
Stamped letters and numbers
(centered on holes)

100 holes
EQ SP (18 mm center to center)
0.5 mm DIA through
13 mm DIA cdrill - 50 mm deep

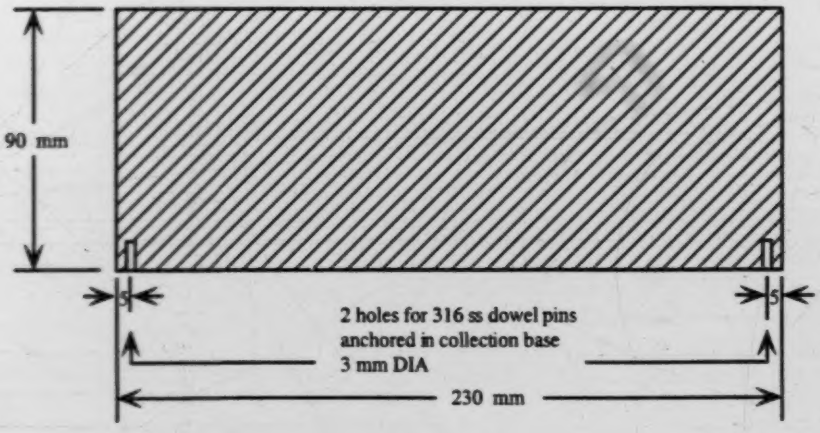
100 REACTOR MODEL

REACTOR BLOCK

SECTION A-A

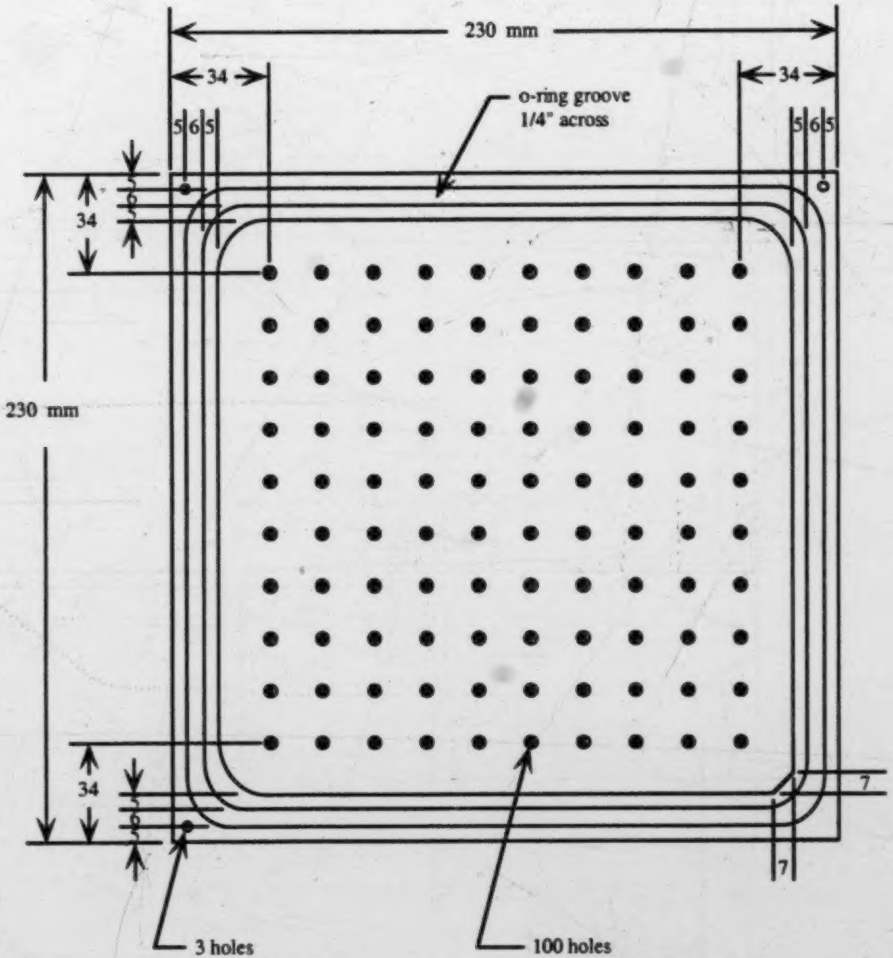

90 mm
50
8
30
11
5
22
100 glass drainage tubes
6 mm OD
0.5 mm ID
o-ring groove
1/4" DIA
1/8" deep
230 mm

SECTION B-B


90 mm
5
5
2 holes for 316 ss dowel pins
anchored in collection base
3 mm DIA
230 mm

100 REACTOR MODEL REACTOR BLOCK

BOTTOM VIEW

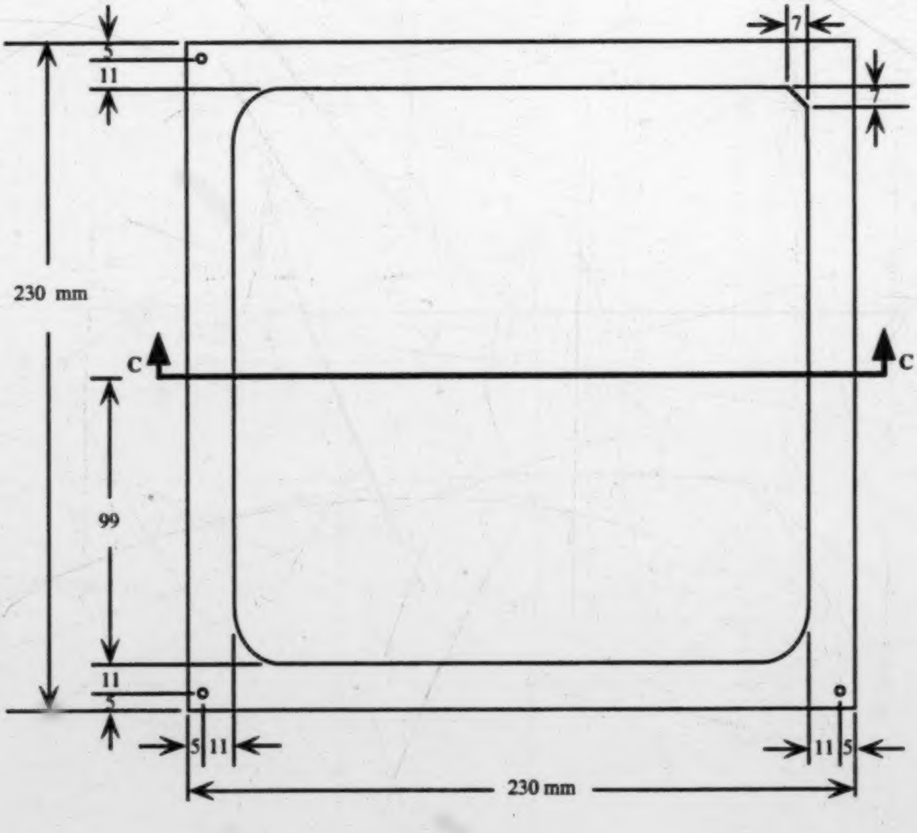

230 mm
34
34
o-ring groove
1/4" across
5 6 5
5 6 5
34
5
6
5
230 mm
34
5
6
5
7
7

3 holes
3 mm DIA - 10 mm deep

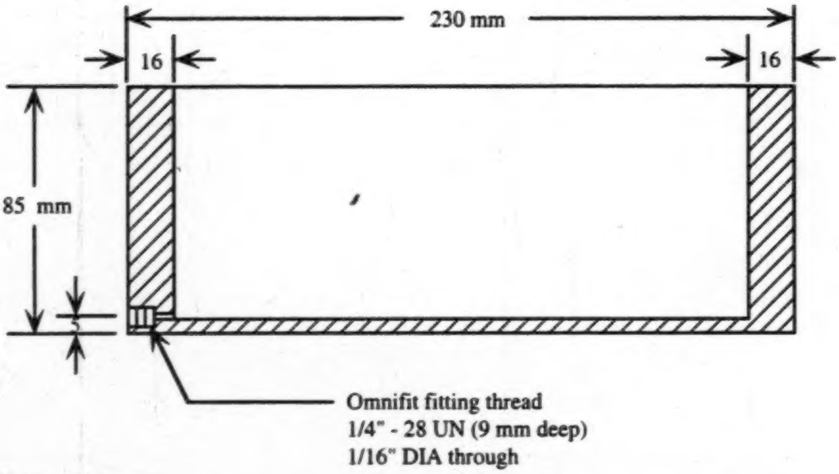
100 holes
EQ SP (18 mm center to center)
0.5 mm DIA through
1.5 mm DIA cdrill - 8 mm deep

COLLECTION BASE

TOP VIEW

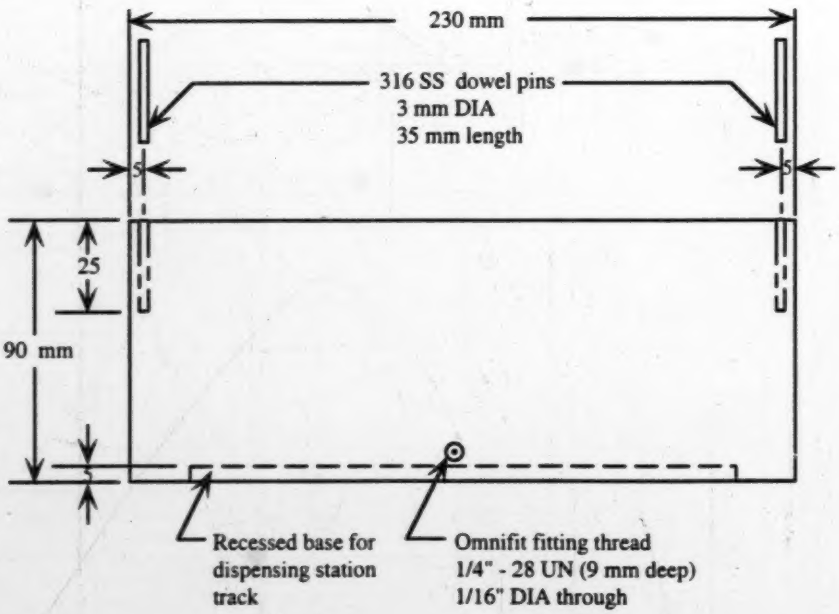

5
11
7
7
230 mm
C
C
99
11
5
5
11
11
5
230 mm

SECTION C-C


230 mm
16
16
85 mm
5

Omnifit fitting thread
1/4" - 28 UN (9 mm deep)
1/16" DIA through

LEFT VIEW

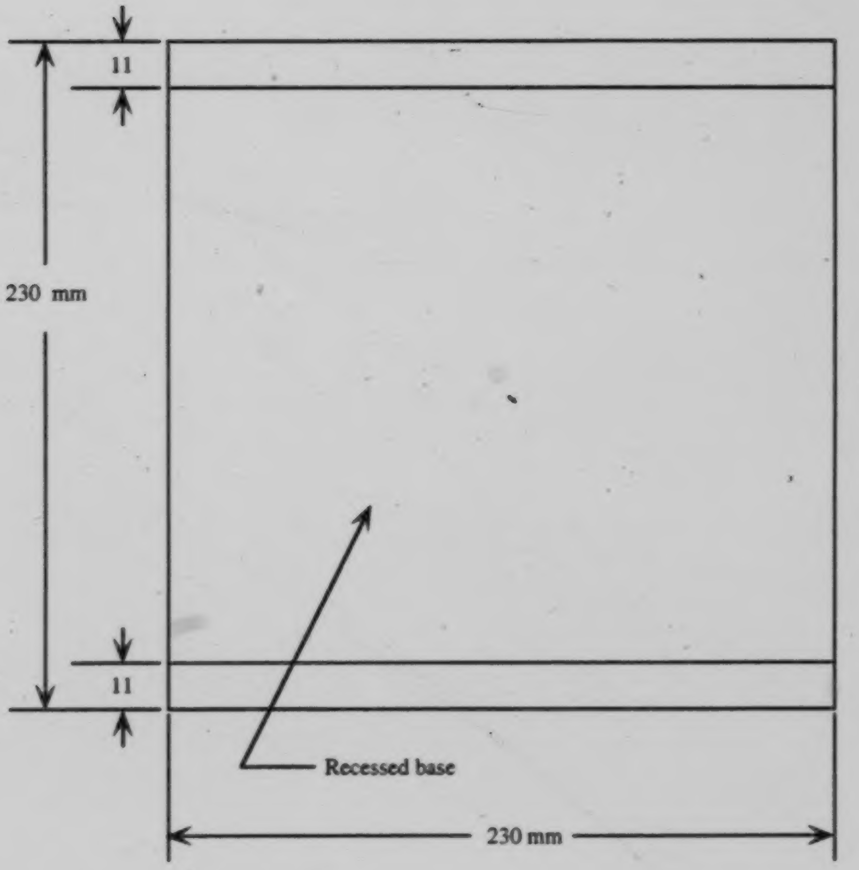

230 mm
5
5
25
90 mm
5

316 SS dowel pins
3 mm DIA
35 mm length

Recessed base for
dispensing station
track

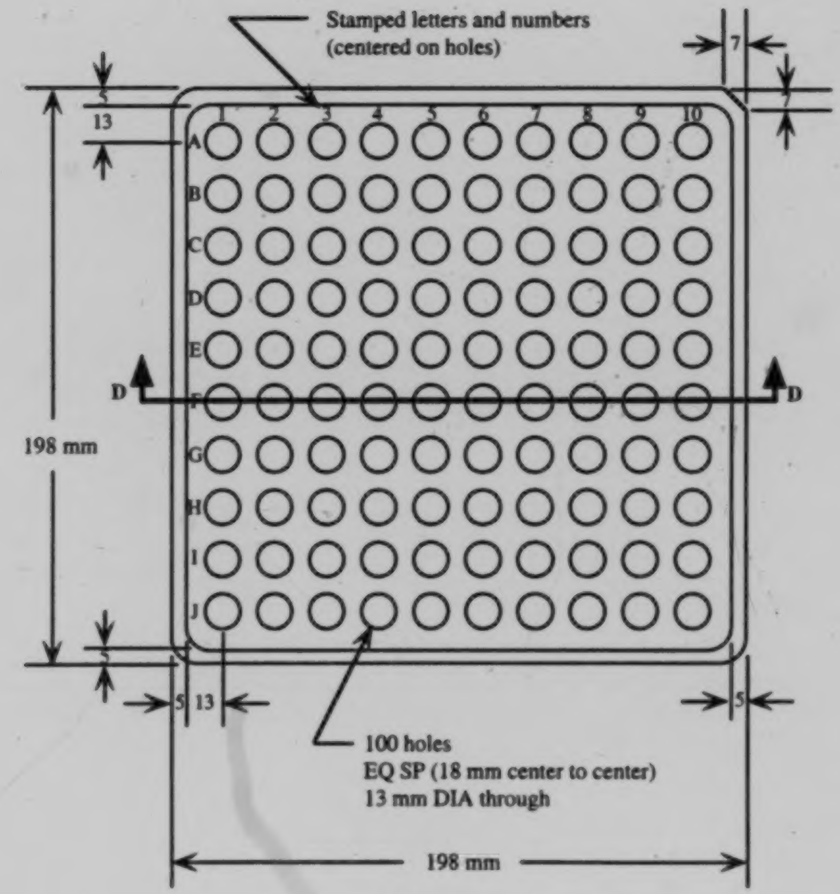
Omnifit fitting thread
1/4" - 28 UN (9 mm deep)
1/16" DIA through

BOTTOM VIEW

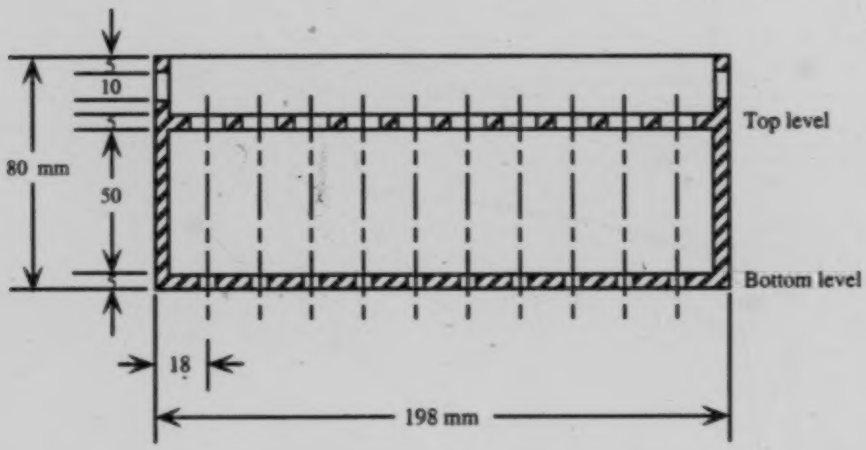

11
230 mm
11
Recessed base
230 mm

100 REACTOR MODEL COLLECTION TUBE RACK

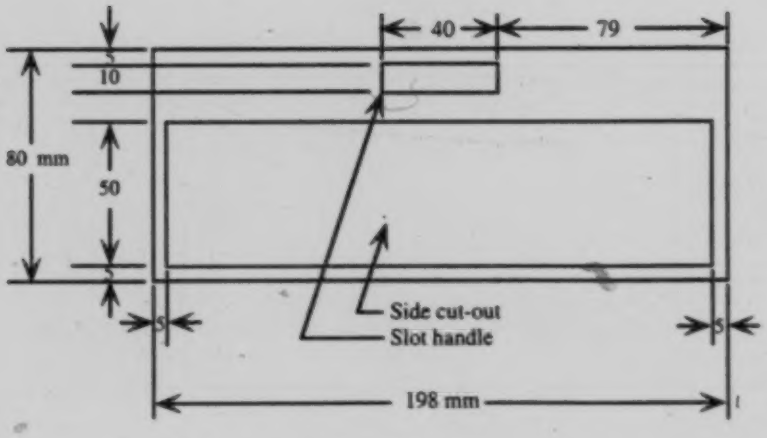
TOP LEVEL


Stamped letters and numbers
(centered on holes)
7
7
5
13
1
2
3
4
5
6
7
8
9
10
A
B
C
D
E
F
G
H
I
J
D
D
198 mm
5
5
13
5
100 holes
EQ SP (18 mm center to center)
13 mm DIA through
198 mm

SECTION D-D

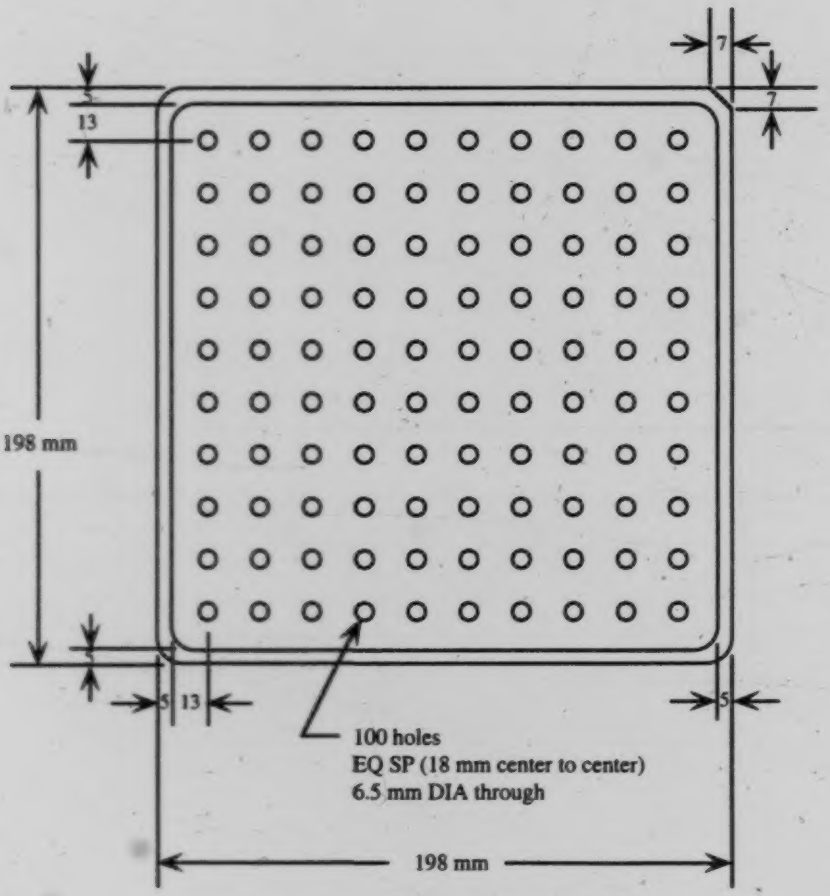

5
10
5
80 mm
50
5
Top level
Bottom level
18
198 mm

LEFT SIDE VIEW


40
79
5
10
80 mm
50
5
5
5
Side cut-out
Slot handle
198 mm

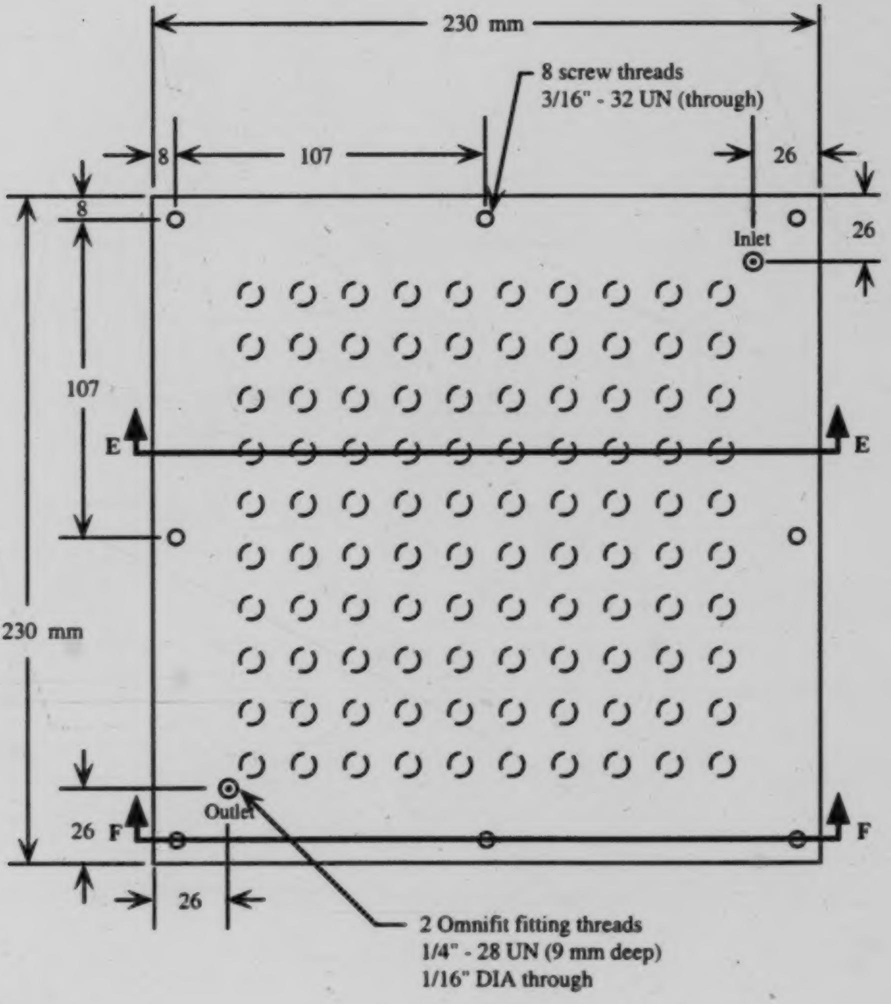
100 REACTOR MODEL COLLECTION TUBE RACK

BOTTOM LEVEL

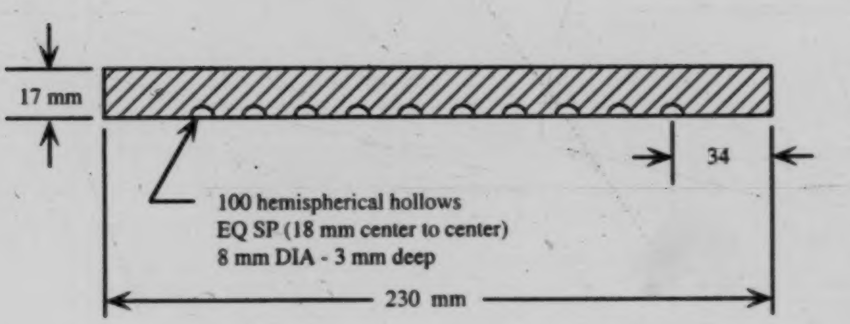

7
7
5
13
198 mm
5
13
5
100 holes
EQ SP (18 mm center to center)
6.5 mm DIA through
198 mm

100 REACTOR MODEL DISPENSER UPPER SECTION

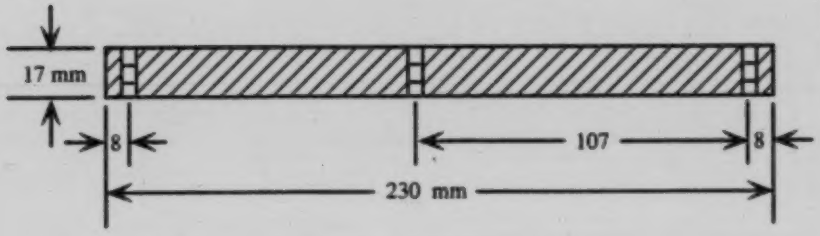
TOP VIEW


230 mm
8 screw threads
3/16" - 32 UN (through)
8
107
26
8
26
Inlet
107
E
E
230 mm
26
F
F
Outlet
26
2 Omnifit fitting threads
1/4" - 28 UN (9 mm deep)
1/16" DIA through

SECTION E-E

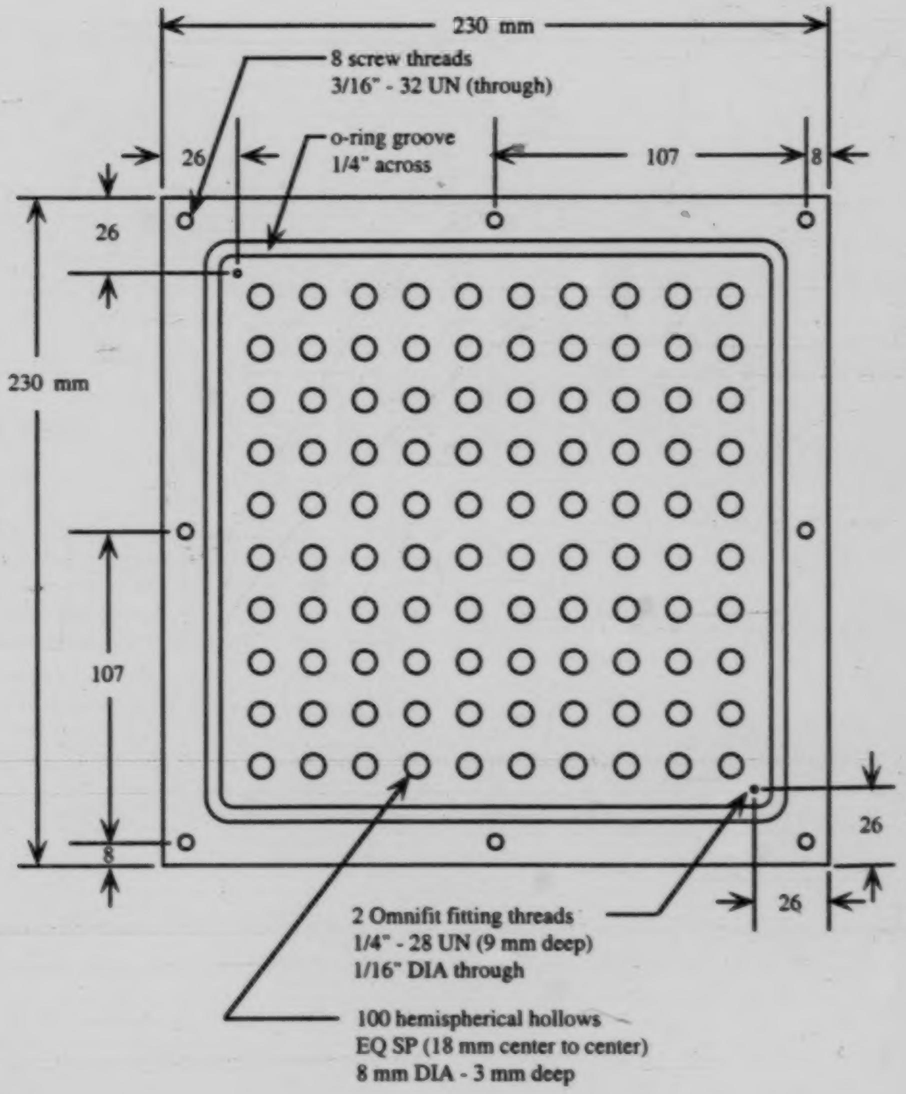

17 mm
34
100 hemispherical hollows
EQ SP (18 mm center to center)
8 mm DIA - 3 mm deep
230 mm

SECTION F-F

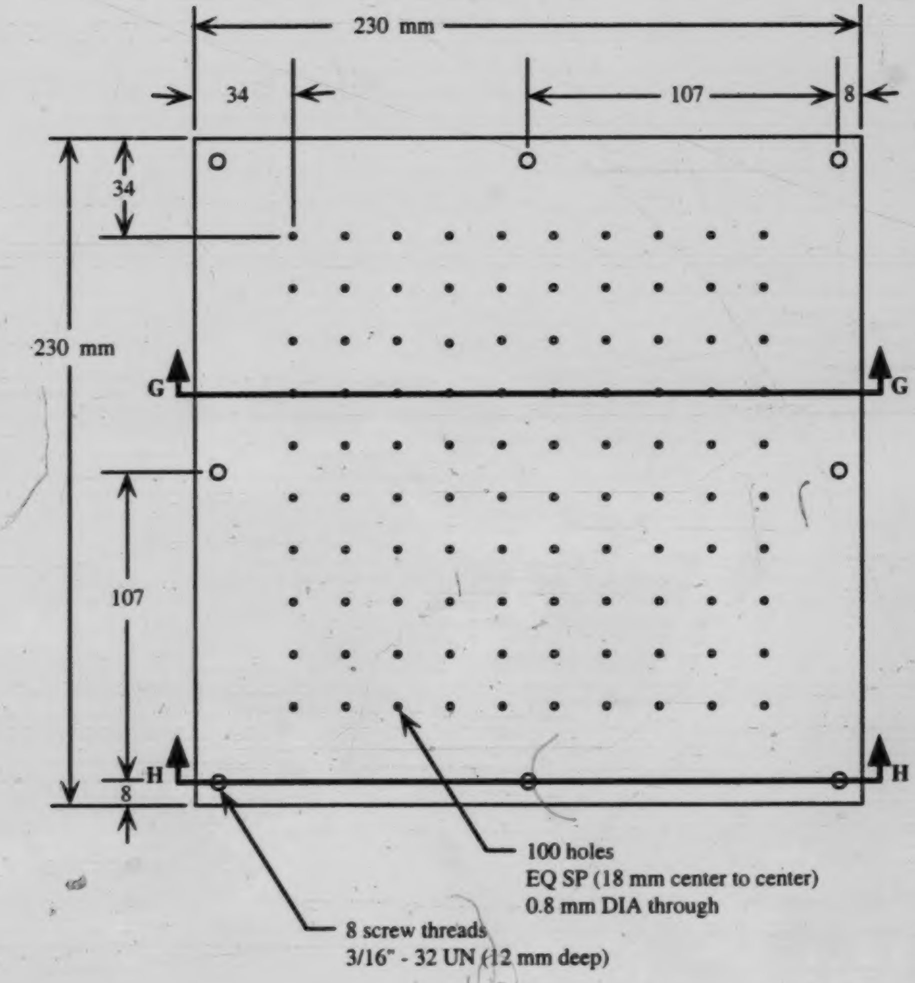

17 mm
8
107
8
230 mm

100 REACTOR MODEL DISPENSER UPPER SECTION

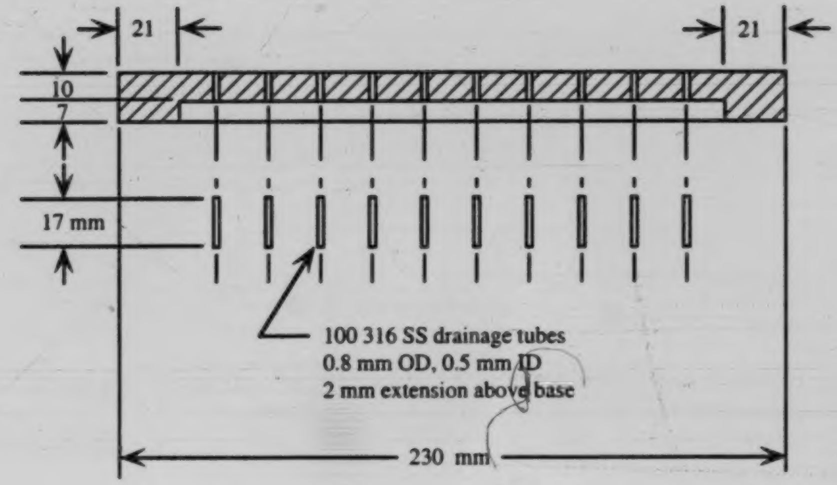
BOTTOM VIEW


230 mm
8 screw threads
3/16" - 32 UN (through)
26
o-ring groove
1/4" across
107
8
26
230 mm
107
8
26
26
2 Omnifit fitting threads
1/4" - 28 UN (9 mm deep)
1/16" DIA through
100 hemispherical hollows
EQ SP (18 mm center to center)
8 mm DIA - 3 mm deep

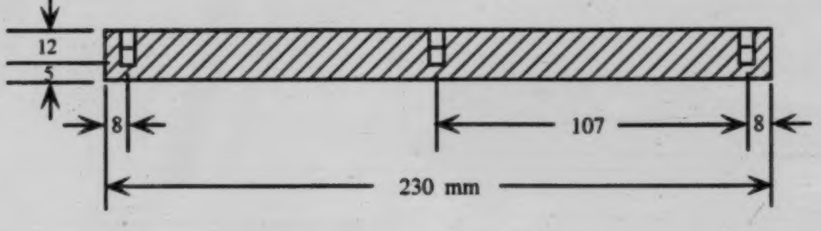
TOP VIEW


230 mm
34
107
8
34
230 mm
G
G
107
H
8
H
100 holes
EQ SP (18 mm center to center)
0.8 mm DIA through
8 screw threads
3/16" - 32 UN (12 mm deep)

SECTION G-G

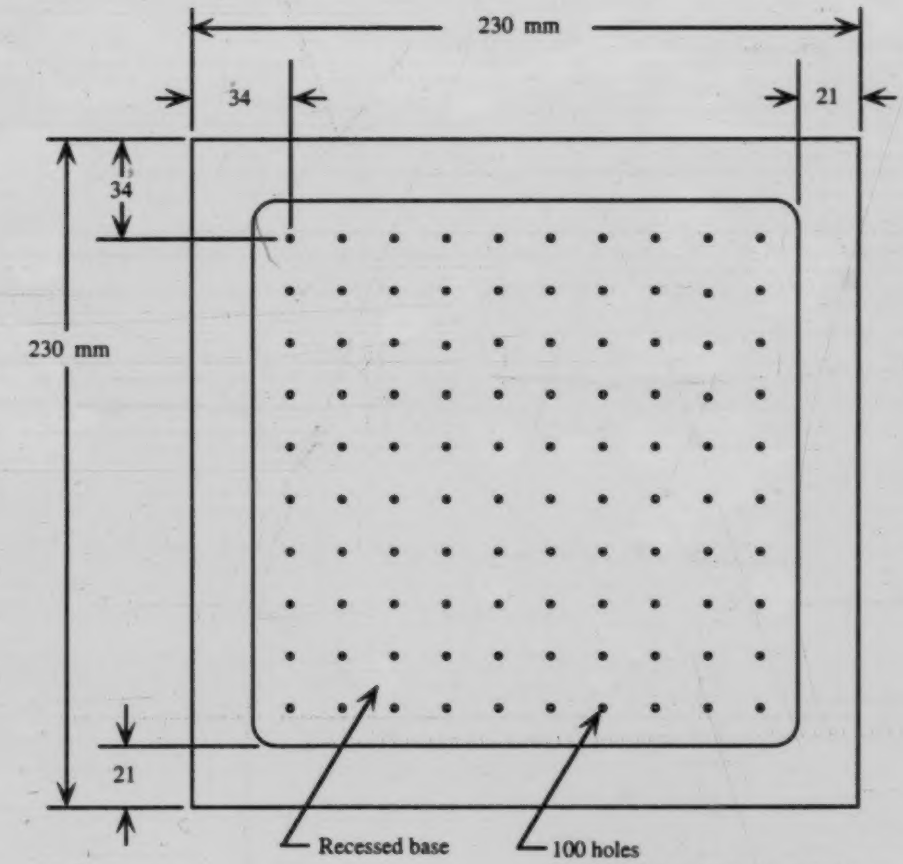

21
21
10
7
17 mm
100 316 SS drainage tubes
0.8 mm OD, 0.5 mm ID
2 mm extension above base
230 mm

SECTION H-H


12
5
8
107
8
230 mm

100 REACTOR MODEL **DISPENSER BASE**

BOTTOM VIEW


230 mm
34
21
34
230 mm
21

Recessed base

100 holes
EQ SP (18 mm center to center)
0.8 mm DIA through

APPENDIX B

MULTIPLE PEPTIDE SYNTHESIZER OPERATING MANUAL

STANDARD PROTOCOL

Protection strategy:	Fmoc	
Resin:	Continuous flow resin	
	(Batch resins may be used, but nitrogen sparging must be employed and a lower quality product may still be the result)	
Solvents:	DMF	(for deprotection, activation, and washing)
	DCM	(for drying resin before cleavage)
Deprotection:	20% piperidine in DMF	(stock solution)
	2 x 10-minute deprotections	
Activation:	*In situ* generation of OBt active esters	
	1: 1: 2: 1 HBTU: HOBt: DIEA: Fmoc-amino acid	(equivalents)
	0.45 M HBTU / HOBt in DMF	(stock solution)
	Fmoc-amino acids dissolved in DMF	(freshly prepared)
	Catalytic base DIEA	(distilled)
Coupling conditions:	Easy coupling	5-fold excess reagents 30 minute coupling time
	Difficult coupling	10-fold excess reagents 60 minute coupling time
Cleavage:	Pre-dry resin using DCM wash Dry under vacuum for at least 1 hour (depending on resin)	
	Modified Reagent K all-purpose cleavage cocktail	
	90% TFA 5% water 3% thioanisole 2% EDT	(freshly prepared)
	2 hour cleavage period	

STANDARD PROTOCOL (continued)

Recovery: Cold ether precipitation of crude peptide from cleavage solution

10:1 volume ratio	(13x100 mm collection test tubes)
20:1 volume ratio	(50 mL conical centrifugation tubes)

Lyophilization

From H_2O (may need up to 10% acetic acid to dissolve)

For use in pH-sensitive assays, may neutralize by lyophilizing from aqueous NH_4HCO_3

STANDARD OPERATING PROCEDURES

INSTALLATION

The Multiple Peptide Synthesizer must be operated in a fumehood due to its open cabinet design. The synthesizer fits into a standard-sized fumehood, which must contain a water tap acting as an aspirator (i.e. vacuum source) for the synthesizer; a nitrogen gas cylinder is required for pressurization of the reaction module.

- Level cabinet according to bubble level on top of dispensing station, by means of adjustable feet.

- Connect aspirator trap to back control panel at labeled connector using provided tubing, then connect trap to aspirator using vacuum tubing; turn on water tap, open evacuation valve at work station valve manifold, and ensure that aspirator is evacuating line leading to aspirator trap, then close valve and turn off tap.

- Connect nitrogen gas cylinder, equipped with pressure reduction valve, to control panel at labeled connector, and adjust reduction valve to achieve a pressure reduction to 15 psi; open reaction module and pressurization valves at work station valve manifold and adjust gas rotameter control valve to achieve a flowrate of 0.1 L/min, then close pressurization valve.

- Select appropriate dispenser module (20- or 100-reactor version), mount it in dispensing station, and connect all lines leading from control panel to dispenser module, as labeled.

- Fill wash and deprotection reservoirs and connect each through a 100 mL bottletop dispenser to control panel at appropriate labeled connector using provided tubing, and set each bottletop dispense to dispense 20 mL (20-reactor model) or 100 mL (100-reactor model); place collection tray at dispensing station.

- Open wash valve at dispensing station valve manifold, test wash dispensing line using bottletop dispenser mounted on wash reservoir, and continue manually dispensing wash solvent until all air has been displaced from dispenser module and each drainage tube is dispensing wash solvent into collection tray below, then close wash valve.

- Repeat above step for deprotection valve and deprotection reservoir, then open wash valve and wash out dispenser module with a minimum of 10 solvent washes (using a wash volume of 20 mL for 20-reactor model or 100 mL for 100-reactor module); close wash valve, empty collection tray to waste and remove.

- Place appropriate reaction module (20- or 100-reactor version) at work station and connect it to control panel at labeled connector using provided tubing.

PREPARATION FOR SYNTHESIS

Synthesis Checkerboards

- Prepare a peptide checkerboard assigning a reactor to each peptide to be synthesized. Reactors are designated by column letter and row number, as seen in the following checkerboard for the 20-reactor model:

A1	B1	C1	D1	E1
A2	B2	C2	D2	E2
A3	B3	C3	D3	E3
A4	B4	C4	D4	E4

- Prepare an amino acid checkerboard for each coupling round of synthesis, assigning the required amino acid residue for each reactor.

Amino Acids

- Select and label 15 mL or 50 mL conical centrifugation tubes, as appropriate, for activated amino acid solutions required for each residue addition to each reactor (common residues can be prepared in a common container).

- Weigh out appropriate amino acids, transfer to their respective containers, and seal and store containers until use.

Preparation of Dispensing Station

- Fill wash reservoir if required.

- Prepare deprotection reagent if required, and fill corresponding reservoir.

- If a different dispenser module (20- or 100-reactor version) desired, disconnect all lines leading to dispenser module, unlock dispenser slot, slide out module and exchange it for desired module, relock dispenser slot, and reconnect all lines as labeled.

Assembly of Reaction Module

- Select desired reaction module (20- or 100-reactor version).
- Disassemble reaction module, and ensure collection base is clean (see Cleaning and Maintenance Procedure if required).
- Ensure column reactors in reactor block are clean; inspect reactor block o-ring and replace if required before proceeding (see Clean-up Procedure).
- Reassemble reaction module, and clamp reactor block and base together.
- Insert bottom frit into each column reactor in block (even unused reactors) using frit installer.
- Vacuum-clean inserted frits and reactor interior as required.

Resin Addition

- For each reactor of reaction module, weigh out appropriate amount of resin and transfer to reactor.

- Load reactors by row or column; tape off currently unused rows or columns to avoid contamination and to keep track of current position in reactor array.

- Cover loaded reactor module to avoid reactor contamination before use.

Resin Swelling

- Before beginning synthesis procedures for the day, resin requires wetting and possible swelling (depending on the resin type).

- Place loaded reaction module on cabinet workspace and connect common inlet / aspiration line from back panel to reaction module. Turn on nitrogen supply to synthesizer (i.e. open gas cylinder valve and adjust pressure as required), open reaction module and pressurization valves at work station valve manifold and adjust gas rotameter control valve as required to achieve a flowrate of 0.1 L/min.

- Slide reaction module into the dispensing station, open wash valve at dispensing station valve manifold, and use bottletop dispenser to dispense appropriate amount of wash solvent for resin scale; turn off wash valve and pressurization valve and leave resin to swell for appropriate time. Once resin swelling is complete, turn on aspirator water supply and open vacuum valve at work station to drain liquid from reactors, then close vacuum valve. Repeat solvent addition and removal as desired to rinse resin, then turn off water supply and remove module from dispensing station.

- At the end of procedure, proceed to synthesis.

SYNTHESIS

Deprotection

- Open reaction module and pressurization valves at work station valve manifold and adjust gas rotameter control valve as required to achieve a flowrate of 0.1 L/min.

- Slide reaction module into dispensing station, open deprotection valve at dispensing station valve manifold, and use bottletop dispenser to dispense appropriate amount of deprotection reagent for resin scale; turn off deprotection valve and pressurization valve and leave resin to deprotect for appropriate time (normally 10 minutes). Turn on aspirator water supply and open vacuum valve at work station to drain liquid from reactors, then close vacuum valve. Repeat deprotection as desired.

- Open pressurization valve at workstation and wash valve at dispensing station, and use bottletop dispenser to dispense appropriate amount of wash solvent for resin scale; turn off wash valve and pressurization valve, open vacuum valve and drain liquid from reactors, then close vacuum valve. Repeat washing as desired (normally 10 times). Remove reaction module from dispensing station and shut off aspirator water supply.

- At end of procedure, proceed to coupling (or if this is final deprotection, proceed to resin drying).

Coupling

- Place amino acid containers for current residue addition in dispensing rack. To simplify manual dispensing, organize containers in rack in arrangement corresponding to reactor array. Prepare activated amino acid solutions, adding reagents to appropriate individual containers in dispensing rack and vortexing.

- Open pressurization valve at work station valve manifold and adjust gas rotameter control valve as required to achieve a flowrate of 0.1 L/min.

- Manual addition of activated amino acids from dispensing rack to reactors is performed by pipet and should proceed by rows or columns; tape off currently unused rows or columns to avoid contamination and to keep track of current position in array of reactors.

- Turn off pressurization valve and allow coupling to proceed for desired time.

- Once complete, turn on aspirator water supply and open vacuum valve to drain liquid from reactors, then close vacuum valve. Slide reaction module into dispensing station for washing (unless a double coupling is required, in which case water supply should be shut off): open pressurization valve, open wash valve and use bottletop dispenser to dispense appropriate amount of wash solvent for resin scale; turn off wash valve and pressurization valve, open vacuum valve and drain liquid from reactors, then close vacuum valve. Repeat washing as desired (normally 10 times). Remove reaction module from dispensing station and shut off aspirator water supply.

- Once complete, continue with next round of deprotection. Dispose of synthesis waste in aspiration trap as required.

Acetylation

- Prepare acetylation solution and fill corresponding reservoir; replace deprotection reservoir connection to dispensing station valve manifold with acetylation reservoir connection. Place collection tray under dispensing station, open deprotection valve and prime acetylation bottletop dispenser until all dispenser tubes are dispensing solution. Remove collection tray and dispose of liquid to waste.

- Open pressurization valve at work station valve manifold and adjust gas rotameter control valve as required to achieve a flowrate of 0.1 L/min.

- Slide reaction module into dispensing station, open deprotection valve at dispensing station valve manifold, and use bottletop dispenser to dispense appropriate amount of acetylation reagent for resin scale; turn off deprotection valve and pressurization valve and leave to acetylate for appropriate time (normally same period as coupling time). Turn on aspirator water supply and open vacuum valve at work station to drain liquid from reactors, then close vacuum valve.

- Open pressurization valve at workstation and wash valve at dispensing station, and use bottletop dispenser to dispense appropriate amount of wash solvent for resin scale; turn off wash valve and pressurization valve, open vacuum valve and drain liquid from reactors, then close vacuum valve. Repeat washing as desired (normally 10 times). Remove reaction module from dispensing station and shut off aspirator water supply.

- Once complete, proceed to resin drying, and dispose of remaining acetylation solution. Dispose of synthesis waste in aspiration trap.

Resin Drying

- Fill drying solvent reservoir (normally DCM). Replace deprotection reservoir connection to dispensing station valve manifold with drying solvent reservoir connection. Place collection tray under dispensing station, open deprotection valve and prime drying solvent bottletop dispenser until all dispenser tubes are dispensing solution. Remove collection tray and dispose of liquid to waste. Use CAUTION when dealing with DCM!

- Turn on aspirator water supply, open pressurization valve at workstation and use bottletop dispenser to dispense appropriate amount of drying solvent for resin scale; turn off deprotection valve and pressurization valve, open vacuum valve and drain liquid from reactors, then close vacuum valve. Repeat washing as desired (normally 3-5 times). Remove reaction module from dispensing station and shut off aspirator water supply.

- Once complete, resins are ready for cleavage. Dispose of synthesis waste in aspirator trap.

PREPARATION FOR CLEAVAGE AND PEPTIDE RECOVERY

Preparation of Reaction Module

- Cover reaction module, disconnect inlet/aspiration line, and carefully disassemble module, placing aside reactor block.
- Clean reaction module base.
- Label cleavage collection tubes by engraving (when dealing with large number of tubes, reactor row-and-column designations are simplest), and place in corresponding positions in cleavage collection tube rack; insert rack into synthesis/cleavage base.
- Reassemble reaction module, clamp reactor block and base together, and reconnect inlet/aspiration line.

Preparation of Dispensing Station

- When using a cleavage solution containing thiol scavengers:
    1. Establish a cleavage waste bottle and a waste collection container within the fumehood for all disposable materials (such as gloves) contaminated with scavengers or cleavage solution.
    2. Double glove before handling scavengers or cleavage solutions.
    3. Bag all disposable materials before removal from the fumehood.

- Prepare cleavage solution and fill corresponding reservoir. Replace deprotection reservoir connection to dispensing station with cleavage reservoir connection. Set bottletop dispenser to displace appropriate amount of cleavage solution for resin scale; place collection tray at dispensing station.

- Open deprotection valve at dispensing station valve manifold and continue manually dispensing cleavage reagent until all air has been displaced from dispenser module and each drainage tube is dispensing solution into collection tray below, then close deprotection valve. Empty collection tray to waste.

Preparation for Peptide Recovery

- If ether precipitation of peptides is to be performed after transfer of cleavage solutions to 50 mL conical centrifugation tubes, label these tubes by engraving (when dealing with large number of tubes, reactor row-and-column designations are simplest), and place in corresponding positions in tube rack.

- Assemble aspirating pipet for removal of supernatant.

- Fill ether reservoir equipped with bottletop dispenser and pre-chill to 4° C. Prepare ice bath for tube rack.

CLEAVAGE

- Remove cover from reaction module.

- Open pressurization valve at work station valve manifold and adjust gas rotameter control valve as required to achieve a flowrate of 0.5 L/min.

- Slide reaction module into dispensing station, open deprotection valve at dispensing station valve manifold, and use bottletop dispenser to dispense appropriate amount of cleavage reagent for resin scale; turn off deprotection valve and reduce pressurization valve to 0.1 L/min, remove reaction module from dispensing station, and leave for appropriate time (normally a 2 hour cleavage period).

- Once cleavage is complete, turn on aspirator water supply and open slightly vacuum valve at work station to assist in draining liquid from reactors into collection test tubes.

- Place reaction module in dispensing station, open deprotection valve and dispense desired cleavage reagent rinse of resins; remove reaction module from dispensing station and leave to drain for 10-15 minutes.

- Immediately after removing reaction module from dispensing station, place collection tray beneath dispenser; open wash valve and dispense wash solvent to rinse out dispenser. Remove collection tray and dispose of contents to waste.

- Once cleavage collection is complete, turn off aspirator water supply. Carefully disassemble reaction module and remove cleavage collection tube rack. Dispose of remaining cleavage solution in cleavage waste bottle. Proceed to peptide recovery.

PEPTIDE RECOVERY (Cold Ether Precipitation)

- If synthesis scale is small ($\leq$ 0.5 mL cleaved peptide solution per collection tube), perform ether precipitation in collection tubes; otherwise, transfer cleaved peptide solutions from collection tubes to 50 mL conical centrifugation tubes by direct manual pouring, rinsing out each collection tube with neat TFA from a dispensing bottle.

- Place collection tube / centrifugation tube rack in ice bath.

- Manually dispense ether from pre-chilled dispensing bottle into tubes in serial fashion, vortexing each tube to ensure complete mixing and using appropriate ether-to-cleaved peptide solution volume ratio:

10:1 ratio	13x100 mm collection test tubes
20:1 ratio	50 mL conical centrifugation tubes

- Leave undisturbed to precipitate for 30 to 60 minutes.

- Remove supernatant from each tube using pipet aspirator, rinsing with ether between each removal.

- Load tubes onto hollow needle evaporator flushed with nitrogen gas for drying and equipped with 30°C water bath to compensate for evaporative cooling; adjust gas flow to each tube using control valves on needles, and allow precipitates to dry completely.

- Crude peptides are prepared for lyophilization by dissolving in minimal amount of water (up to 10% acetic acid may be required to solubilize peptides); lyophilization from NH_4HCO_3 (aq) can be performed if pH adjustment is required.

CLEANING AND MAINTENANCE

Reaction Module

- If scavengers were used in cleavage solution, reaction module will require bleaching to remove resulting odor.

- Up-end reactor block within waste collection bag and strike sharply to dislodge resin. Remove reactor frits using frit removal tool, and vacuum-clean reactors of remaining solid material (using solid collection trap).

- Soak reactor block, synthesis/cleavage base, and cleavage collection tube rack in bleach bath.

- If reactors exhibit staining, reassemble reaction module, reconnect inlet/aspiration line, pressurize base, fill reactors with 25-50% nitric acid, and soak overnight.

- Wash reaction module and cleavage collection tube rack in soapy water, thorougly rinse, and dry overnight.

- Insert new o-ring in reactor block after every cleavage; inspect reactor drainage tubes for blockage and corrosion, and clean or replace as required.

Dispenser

- If scavengers were used in cleavage solution, dispenser module will require bleaching to remove resulting odor.

- Remove dispenser module from dispensing station, disassemble module, remove o-ring, and soak top and bottom blocks in bleach bath.
- Wash dispenser module in soapy water, thorougly rinse, and dry overnight.
- Insert new o-ring in top block; inspect dispensing tubes in bottom block for blockage and corrosion, and clean or replace as required.

Cabinet

- Periodically inspect all tubing and connectors for blockage or leaks, and clean with acetone or replace as required.
- Periodically inspect reservoir bottletop dispensers for blockage or leaks, and clean with ethanol as required; a crystalline deposit may build up in the deprotection reservoir dispenser if left idle, and must be removed before resuming use.

APPENDIX C

SEMI-AUTOMATED MULTIPLE PEPTIDE SYNTHESIZER (S.A.M.P.S.)

Parts list and specifications
Automated functions
Programming displays

S.A.M.P.S. PARTS LIST AND SPECIFICATIONS

Open cabinet:

Dispensing station

5 peristaltic dispenser pumps
(mounted on back panel)

Nitrogen gas rotameter with control valve
(mounted on back panel)

Aspirator trap (with vacuum tubing)

Power port and on/off switch
Dispenser LCD controller port

Dispensing station:

Slide-in ledge for interchangeable dispensers
Waste collection tray
Slide-in track for interchangeable reaction modules

20-reactor dispenser module
100-reactor dispenser module

Hand-held LCD controller with cable

Solvent reservoirs (with connectors): 3 x 4 L
2 x 500 ml

Reaction modules:

20-reactor reaction module
- Cover
- Cleavage collection tube rack (insert)

100-reactor reaction module
- Cover
- Cleavage collection tube rack (insert)

Accessories:

Frit installer (tool)
Frit remover (tool)
Dispensing rack for activated amino acid solutions

Replacement parts:

Reactor block drainage tubes (threaded)
Dispensing tubes (threaded)

S.A.M.P.S. PARTS LIST AND SPECIFICATIONS (continued)

Disposables:		
	Reactor bottom frits	
	Reaction module and dispenser module o-rings	
	Pump tubing	
	Reaction module connection tubing	
	Activated amino acid solution tubes	(15 mL and 50 mL)
	Cleavage collection test tubes	(13 x 100 mm)
	Precipitation conical centrifuge tubes	(50 mL)

Overall specifications:		
	Width	82 cm (32 in.)
	Height	44 cm (17 in.)
	Depth	41 cm (16 in.)
	Weight	36 kg (80 lb.)
	Site	Standard fumehood
	Electrical	110 V / 220 V
	Other requirements:	Nitrogen gas supply (cylinder) Water tap with aspirator

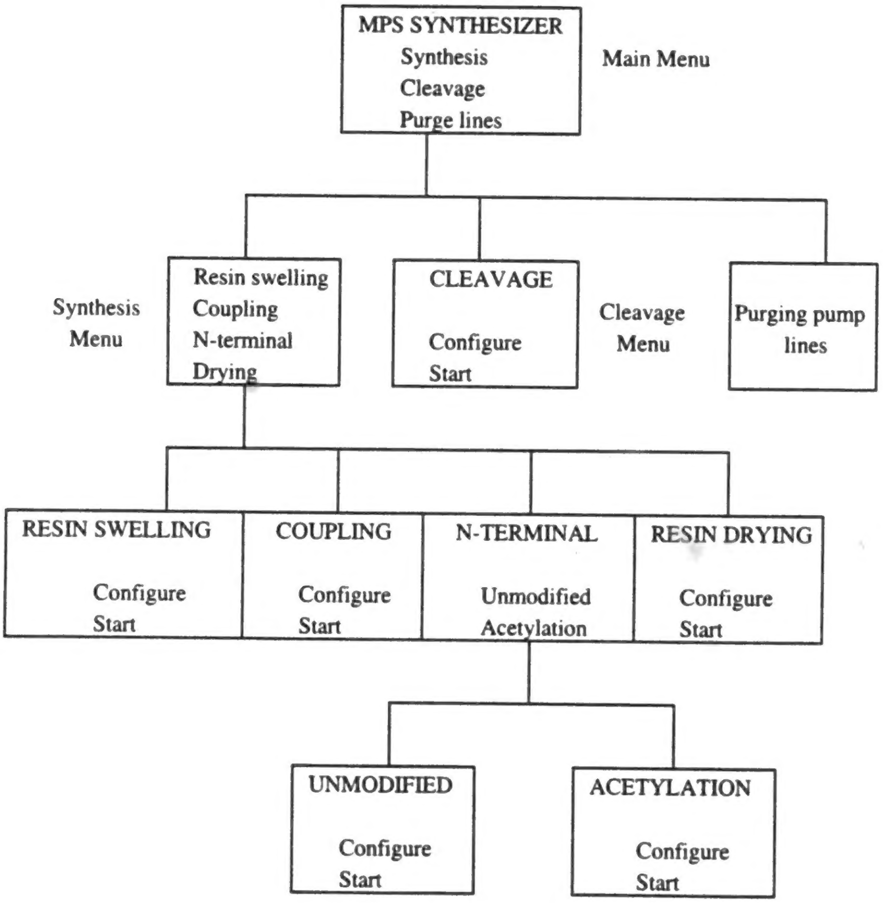
AUTOMATED FUNCTIONS

S.A.M.P.S. automated functions are the common synthesis steps of washing, deprotection, N-terminal modification (such as acetylation), and cleavage, as well as housekeeping functions such as pump line priming and purging. The addition of activated amino acid solutions for coupling must be performed manually, and this manual option is also available for acetylation (in case acetylation is not required for all peptides in the reaction module) and cleavage (in case different cleavage solutions are desired for different peptides). The automated functions are performed by a microprocessor-controlled dispenser with interactive programming based on menu-driven selection of functions and modification of function parameters; the functions are independent, permitting a synthesis to be performed in discrete stages rather than in an uninterruptable sequence.

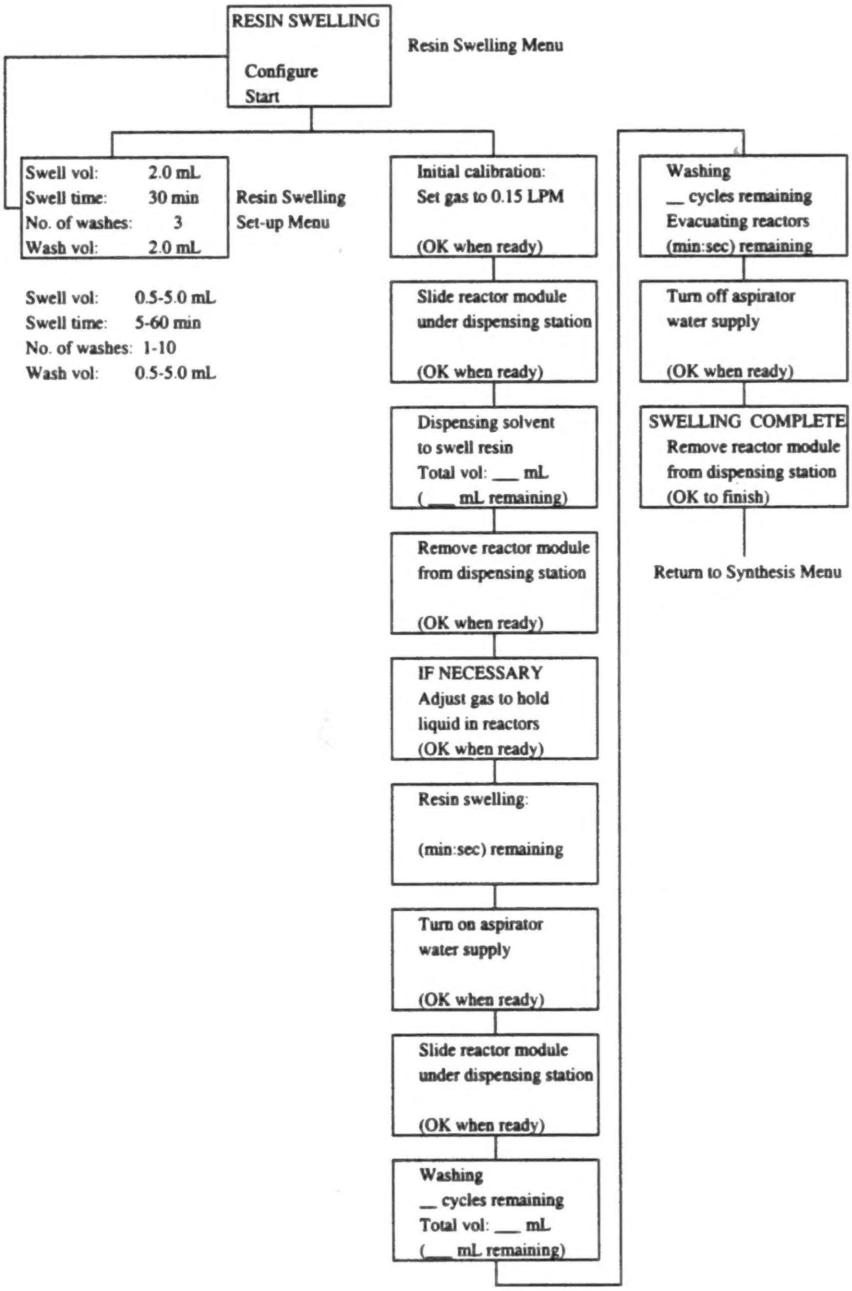
Programming details are provided as flowsheets in the following pages, and include menus, parameter ranges for the different functions, and recommended parameter values. The **Main Menu** provides access to **[Synthesis]**, **[Cleavage]**, and **[Line purging]** options; synthesis functions are performed independently and consist of **Resin Swelling**, **Coupling** (including deprotection and washing steps), **N-terminal** (with a choice of **Final Deprotection** or an N-terminal modification such as **Acetylation**), and **Resin Drying**. Menu selections are accessed by arrow keys and selected by the **OK** key, while the **ESC** key returns the user to the previous menu. Function menus provide access to parameters through the **[Configure]** option, and allow the function to be initiated through the **[Start]** option. Parameters are selected by arrow keys, old values are cleared by the **CE** key, and new values are entered by the **OK** key. It is important to note that each function retains the latest parameter values upon shutdown, for use as default values upon start-up. Once a function has been initiated, the procedure can be paused by use of the **ESC** key; a second use of the **ESC** key aborts the process (in case of an error), while the **OK** key resumes the procedure.

PROGRAMMING DISPLAYS

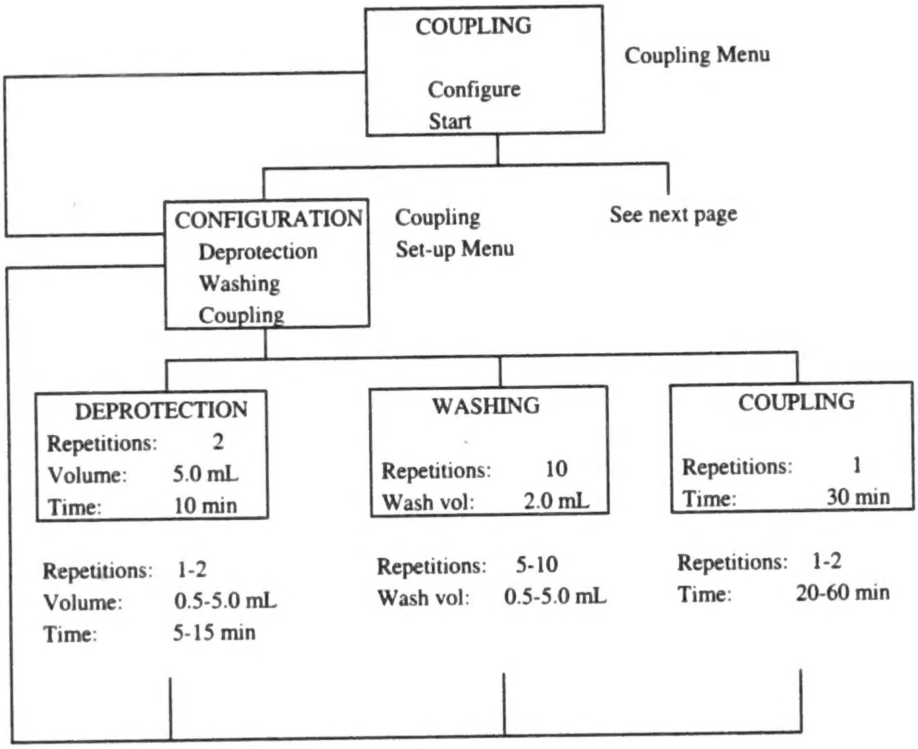
Organizational Overview

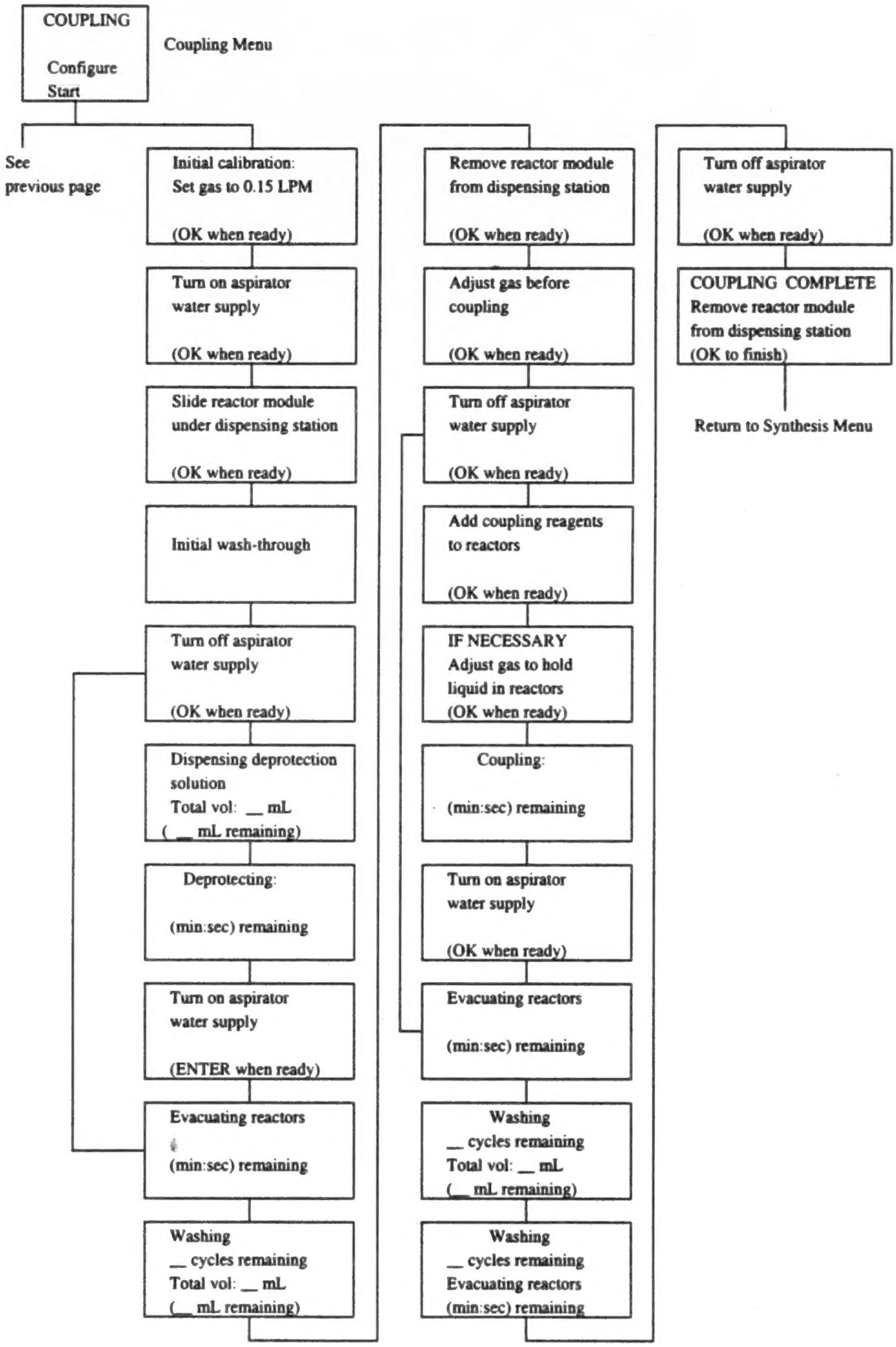

MPS SYNTHESIZER
Synthesis
Cleavage
Purge lines
Main Menu
Synthesis Menu
Resin swelling
Coupling
N-terminal
Drying
CLEAVAGE
Configure
Start
Cleavage Menu
Purging pump lines
RESIN SWELLING
Configure
Start
COUPLING
Configure
Start
N-TERMINAL
Unmodified
Acetylation
RESIN DRYING
Configure
Start
UNMODIFIED
Configure
Start
ACETYLATION
Configure
Start

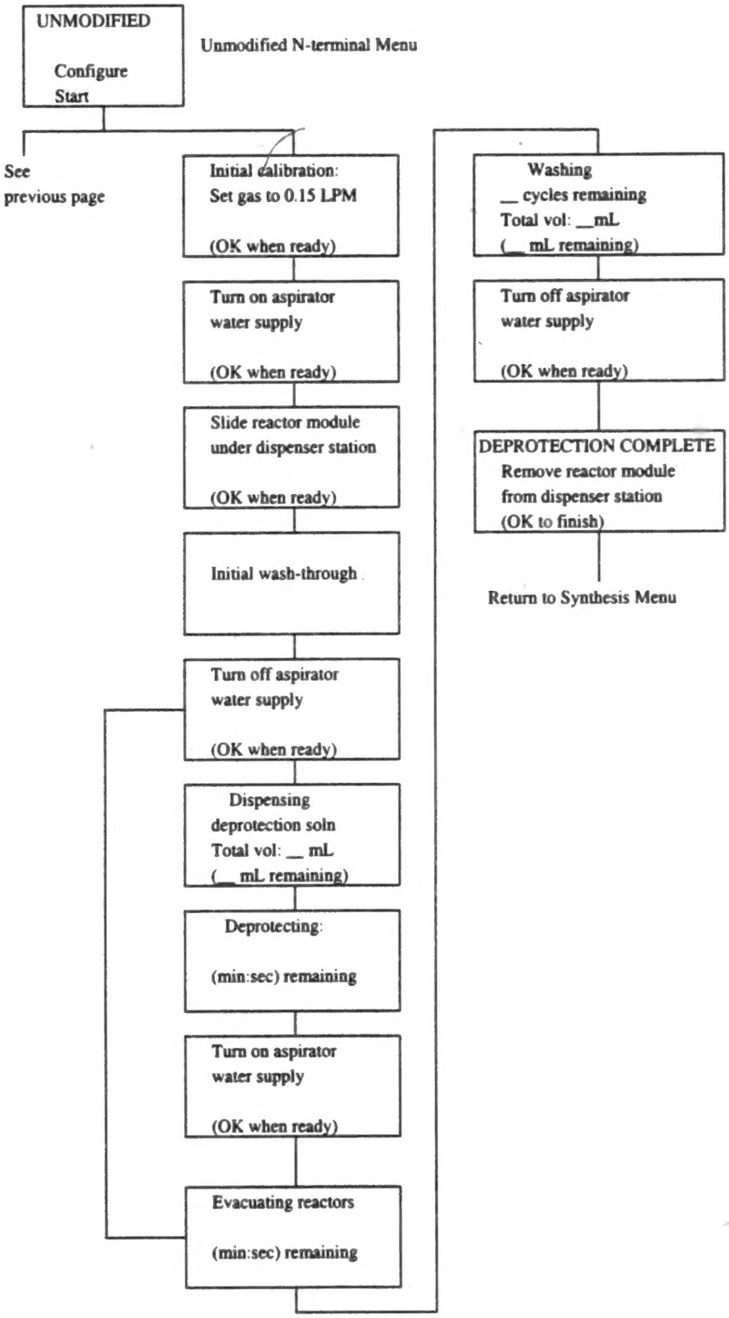
Resin Swelling Function

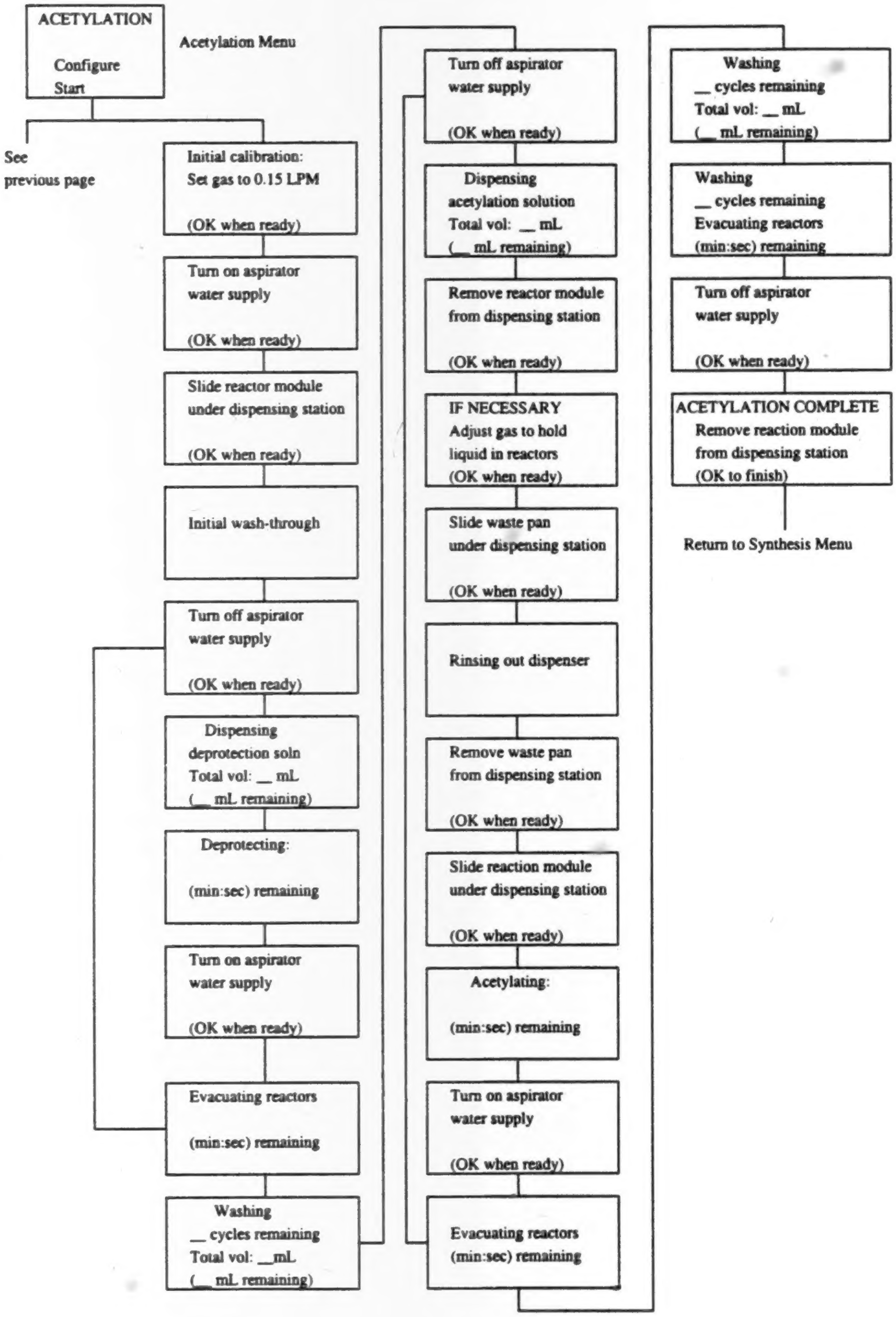

RESIN SWELLING
Configure
Start
Resin Swelling Menu
Swell vol: 2.0 mL
Swell time: 30 min
No. of washes: 3
Wash vol: 2.0 mL
Resin Swelling Set-up Menu
Swell vol: 0.5-5.0 mL
Swell time: 5-60 min
No. of washes: 1-10
Wash vol: 0.5-5.0 mL
Initial calibration: Set gas to 0.15 LPM (OK when ready)
Slide reactor module under dispensing station (OK when ready)
Dispensing solvent to swell resin Total vol: ___ mL (___ mL remaining)
Remove reactor module from dispensing station (OK when ready)
IF NECESSARY Adjust gas to hold liquid in reactors (OK when ready)
Resin swelling: (min:sec) remaining
Turn on aspirator water supply (OK when ready)
Slide reactor module under dispensing station (OK when ready)
Washing __ cycles remaining Total vol: ___ mL (___ mL remaining)
Washing __ cycles remaining Evacuating reactors (min:sec) remaining
Turn off aspirator water supply (OK when ready)
SWELLING COMPLETE Remove reactor module from dispensing station (OK to finish)
Return to Synthesis Menu

Coupling Function

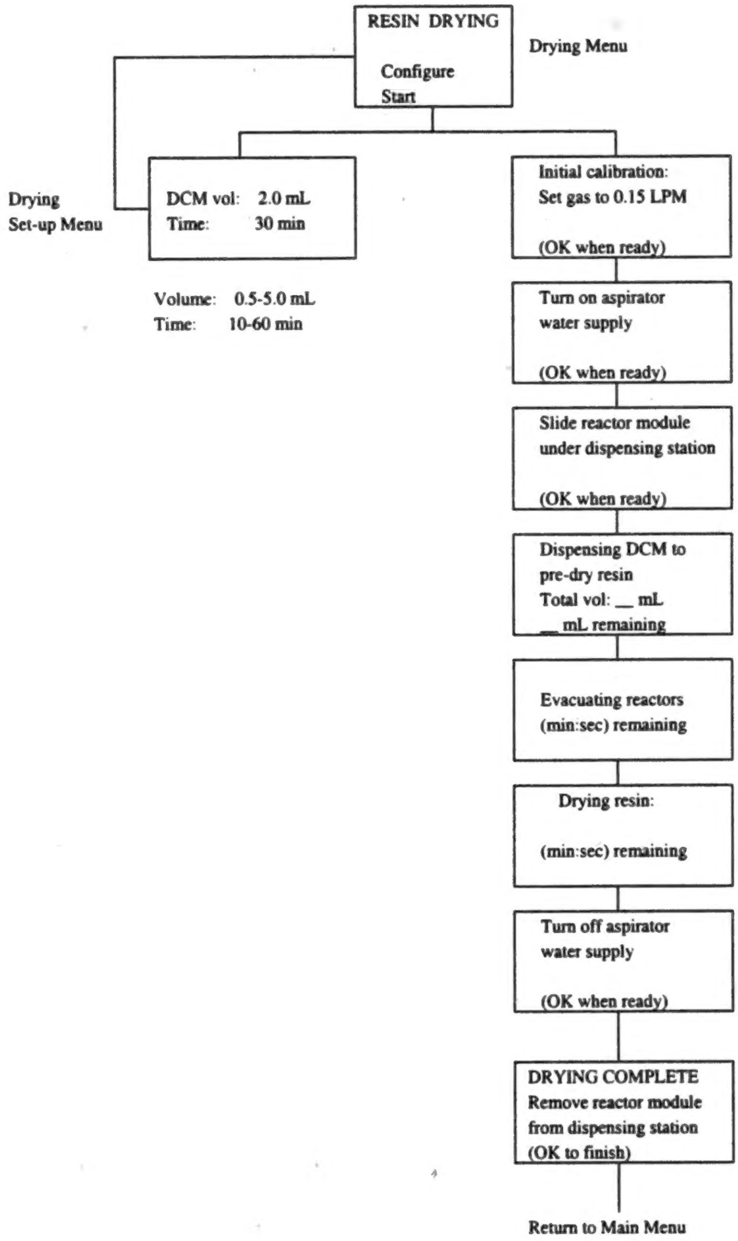

COUPLING
Configure
Start
Coupling Menu
CONFIGURATION
Deprotection
Washing
Coupling
Coupling
Set-up Menu
See next page
DEPROTECTION
Repetitions: 2
Volume: 5.0 mL
Time: 10 min
WASHING
Repetitions: 10
Wash vol: 2.0 mL
COUPLING
Repetitions: 1
Time: 30 min
Repetitions: 1-2
Volume: 0.5-5.0 mL
Time: 5-15 min
Repetitions: 5-10
Wash vol: 0.5-5.0 mL
Repetitions: 1-2
Time: 20-60 min


COUPLING
Configure
Start
Coupling Menu
See previous page
Initial calibration: Set gas to 0.15 LPM (OK when ready)
Turn on aspirator water supply (OK when ready)
Slide reactor module under dispensing station (OK when ready)
Initial wash-through
Turn off aspirator water supply (OK when ready)
Dispensing deprotection solution Total vol: __ mL (__ mL remaining)
Deprotecting: (min:sec) remaining
Turn on aspirator water supply (ENTER when ready)
Evacuating reactors (min:sec) remaining
Washing __ cycles remaining Total vol: __ mL (__ mL remaining)
Remove reactor module from dispensing station (OK when ready)
Adjust gas before coupling (OK when ready)
Turn off aspirator water supply (OK when ready)
Add coupling reagents to reactors (OK when ready)
IF NECESSARY Adjust gas to hold liquid in reactors (OK when ready)
Coupling: (min:sec) remaining
Turn on aspirator water supply (OK when ready)
Evacuating reactors (min:sec) remaining
Washing __ cycles remaining Total vol: __ mL (__ mL remaining)
Washing __ cycles remaining Evacuating reactors (min:sec) remaining
Turn off aspirator water supply (OK when ready)
COUPLING COMPLETE Remove reactor module from dispensing station (OK to finish)
Return to Synthesis Menu

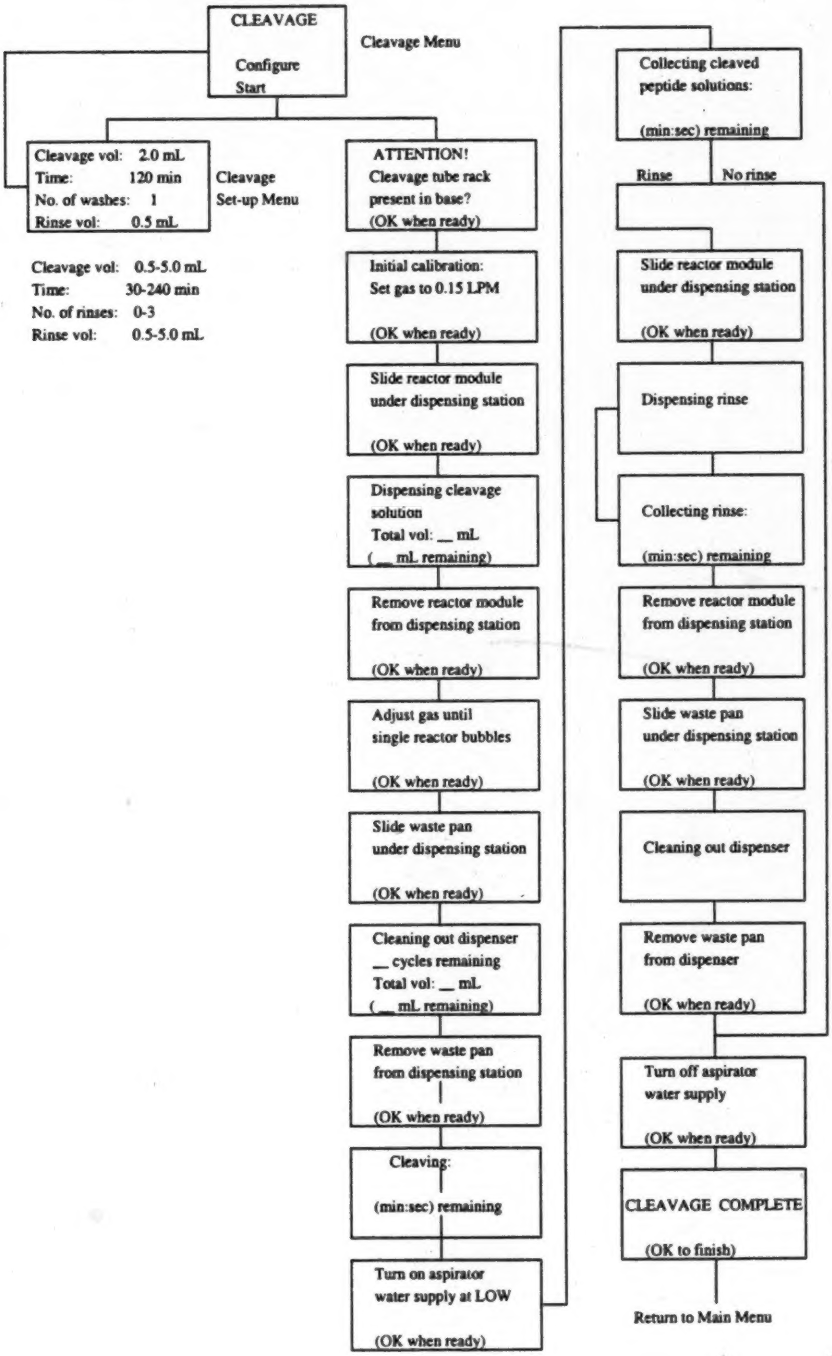

UNMODIFIED
Configure
Start
Unmodified N-terminal Menu
See previous page
Initial calibration:
Set gas to 0.15 LPM
(OK when ready)
Turn on aspirator water supply
(OK when ready)
Slide reactor module under dispenser station
(OK when ready)
Initial wash-through
Turn off aspirator water supply
(OK when ready)
Dispensing deprotection soln
Total vol: __ mL
(__ mL remaining)
Deprotecting:
(min:sec) remaining
Turn on aspirator water supply
(OK when ready)
Evacuating reactors
(min:sec) remaining
Washing
__ cycles remaining
Total vol: __mL
(__ mL remaining)
Turn off aspirator water supply
(OK when ready)
DEPROTECTION COMPLETE
Remove reactor module from dispenser station
(OK to finish)
Return to Synthesis Menu


ACETYLATION
Configure
Start
Acetylation Menu
See previous page
Initial calibration: Set gas to 0.15 LPM (OK when ready)
Turn on aspirator water supply (OK when ready)
Slide reactor module under dispensing station (OK when ready)
Initial wash-through
Turn off aspirator water supply (OK when ready)
Dispensing deprotection soln Total vol: __ mL (__ mL remaining)
Deprotecting: (min:sec) remaining
Turn on aspirator water supply (OK when ready)
Evacuating reactors (min:sec) remaining
Washing __ cycles remaining Total vol: __mL (__ mL remaining)
Turn off aspirator water supply (OK when ready)
Dispensing acetylation solution Total vol: __ mL (__ mL remaining)
Remove reactor module from dispensing station (OK when ready)
IF NECESSARY Adjust gas to hold liquid in reactors (OK when ready)
Slide waste pan under dispensing station (OK when ready)
Rinsing out dispenser
Remove waste pan from dispensing station (OK when ready)
Slide reaction module under dispensing station (OK when ready)
Acetylating: (min:sec) remaining
Turn on aspirator water supply (OK when ready)
Evacuating reactors (min:sec) remaining
Washing __ cycles remaining Total vol: __ mL (__ mL remaining)
Washing __ cycles remaining Evacuating reactors (min:sec) remaining
Turn off aspirator water supply (OK when ready)
ACETYLATION COMPLETE Remove reaction module from dispensing station (OK to finish)
Return to Synthesis Menu

Resin Drying Function


RESIN DRYING
Configure
Start
Drying Menu
Drying
Set-up Menu
DCM vol: 2.0 mL
Time: 30 min
Volume: 0.5-5.0 mL
Time: 10-60 min
Initial calibration:
Set gas to 0.15 LPM
(OK when ready)
Turn on aspirator
water supply
(OK when ready)
Slide reactor module
under dispensing station
(OK when ready)
Dispensing DCM to
pre-dry resin
Total vol: __ mL
__ mL remaining
Evacuating reactors
(min:sec) remaining
Drying resin:
(min:sec) remaining
Turn off aspirator
water supply
(OK when ready)
DRYING COMPLETE
Remove reactor module
from dispensing station
(OK to finish)
Return to Main Menu

Cleavage Function


CLEAVAGE
Configure
Start
Cleavage Menu
Cleavage vol: 2.0 mL
Time: 120 min
No. of washes: 1
Rinse vol: 0.5 mL
Cleavage
Set-up Menu
Cleavage vol: 0.5-5.0 mL
Time: 30-240 min
No. of rinses: 0-3
Rinse vol: 0.5-5.0 mL
ATTENTION!
Cleavage tube rack
present in base?
(OK when ready)
Initial calibration:
Set gas to 0.15 LPM
(OK when ready)
Slide reactor module
under dispensing station
(OK when ready)
Dispensing cleavage
solution
Total vol: __ mL
(__ mL remaining)
Remove reactor module
from dispensing station
(OK when ready)
Adjust gas until
single reactor bubbles
(OK when ready)
Slide waste pan
under dispensing station
(OK when ready)
Cleaning out dispenser
__ cycles remaining
Total vol: __ mL
(__ mL remaining)
Remove waste pan
from dispensing station
(OK when ready)
Cleaving:
(min:sec) remaining
Turn on aspirator
water supply at LOW
(OK when ready)
Collecting cleaved
peptide solutions:
(min:sec) remaining
Rinse
No rinse
Slide reactor module
under dispensing station
(OK when ready)
Dispensing rinse
Collecting rinse:
(min:sec) remaining
Remove reactor module
from dispensing station
(OK when ready)
Slide waste pan
under dispensing station
(OK when ready)
Cleaning out dispenser
Remove waste pan
from dispenser
(OK when ready)
Turn off aspirator
water supply
(OK when ready)
CLEAVAGE COMPLETE
(OK to finish)
Return to Main Menu